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13. ABSTRACT *(Maximum 200*

This study assesses the health risks associated with embedded depleted uranium (DU) fragments by evaluating the behavioral, physiological and histological consequences of intramuscularly implanted DU pellets in a rodent model. Animals, distributed into 5 experimental groups [1) control (20 1-mmx2-mm chemically inert tantalum (Ta) pellets), 2) high dose (20 1-mmx2-mm DU pellets), 3) medium dose (10 DU and 10 Ta pellets), 4) low dose (4 DU and 16 Ta pellets) and 5) nonsurgical controls] were analyzed at the 30 day and 6 month time points. Examination of the pellets *in situ* reveals fibrous tissue adhering to the DU but not the Ta pellets. Uranium levels are high and dose-dependent in kidney, urine, and bone. Despite high uranium levels in kidney, no renal toxicity was evident. Between 23-26 weeks, body weight in high-DU dose animals was significantly lower than controls. Unexpectedly, uranium was found in the brain of DU-implanted animals. No behavioral neurotoxicity was evident. However, excitability of hippocampal neurons was reduced in the high DU dose animals at 6 months. These data suggest that renal toxicity may be less of a hazard than anticipated but that cognitive deficits need to be considered. The 12 and 18 month time points will be examined in future experiments.

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INTRODUCTION

Natural uranium consists of three isotopes: ^{238}U (99.276%), ^{235}U (0.718%) and ^{234}U (0.0056%). During the uranium enrichment process two products are produced, "enriched uranium" and "depleted uranium" (DU), that contain different relative ratios of these three isotopes. Enriched uranium contains the higher amount of the fissionable isotope ^{235}U and is used for nuclear reactor fuel and nuclear weapons. DU has a lower ^{235}U content and is a highly dense material. The DU used by the US in kinetic energy penetrators is alloyed with titanium (0.75% by weight) to retard oxidation. This DU alloy is of concern because the U.S. military currently uses this metal for munitions and armament. During Operation Desert Storm, a number of U.S. military personnel were wounded by shrapnel fragments consisting of DU[6,7]. Since surgical removal can produce excessive tissue damage, these DU fragments were treated as conventional shrapnel and left in place in the wounded soldiers. The radiographs of injured soldiers show multiple embedded fragments ranging in size from 1 mm to over 5 mm in diameter. Fragments as large as 20 mm have been noted in other patients. Uranium bioassays taken over a year after injury indicate that uranium was present in the urine well in excess of natural background, up to 30 μg U/l of urine. DU fragments present a radiologically and toxicologically unique situation with unknown health risks. Congress has mandated the study of these risks.

This study evaluates the consequences of both short-term and long-term exposure to DU fragments in the rat model. Using an interdisciplinary approach, we are assessing neurotoxicity, nephrotoxicity, histopathology of the tissue surrounding the fragment and pathology including evaluation of neoplastic changes in several body tissues. In addition, based on our animal data, we will develop a biokinetic model that describes the distribution of uranium from embedded fragments as a function of time.

Uranium toxicity: Although the toxicity of embedded DU is unknown, numerous studies have addressed the consequences of inhalation, ingestion and parenteral administration of other forms of uranium[27,38,45,62]. After uranium is absorbed, it circulates in the blood as the uranyl ion forming uranium-carbonate and uranium-albumin complexes[8,26,31]. As the uranium-carbonate complex passes through the kidney, it is filtered rapidly at the glomerulus where 60%-80% of absorbed uranium is excreted in the first 24 hours after acute exposure. The uranium not excreted is reabsorbed by the proximal tubules where it produces acute toxic effects. Uranium also enters the bone where it competes with calcium to form complexes with phosphate ions, thus becoming part of the bone matrix[3,10,16,42]. This bone matrix then serves as a storage site from which uranium is slowly released back into circulation [23,61]. The liver, muscle, and kidney are other major sites of uranium disposition, with a possible long-term storage mechanism in the kidney [19,23,27,51,62]. At low doses, uranium may not readily distribute to the central nervous system (CNS)[45]. With higher doses (8 mg/kg/day orally for 4 weeks), however, brain uranium levels are comparable to those in liver and in bone[45], major sites for uranium accumulation.

Acute morphological and biochemical changes of the kidney result from uranium exposure[8,26,31,42]. The glomerular epithelial architecture is altered[25] and cellular necrosis occurs in the proximal tubules near the corticomedullary junction in the kidney[2,17,18]. In addition, polyuria, enzymuria, glucosuria, and increased excretion of amino acids result[8,9,26,63]. Acute renal failure can be the cause of death with exposure to high doses of either soluble or insoluble forms of uranium[43,57]. Environmental stressors such as restricted diets or changes in housing conditions significantly enhance uranium toxicity[1,4].

Few studies have addressed the chronic toxicity of uranium and the results available are conflicting. Galibin and colleagues[14] reported severe renal toxicity in rats that inhaled the slightly soluble uranium

compound, ammonium diuranate (1 or 8 mg/m^3) for 128 days. Urine protein and blood, non-protein nitrogen were elevated. In the proximal tubules, there were sloughed dead cells and abnormal regenerating cells. These animals recovered, although the total number of tubules was reduced, with an accompanying increased proportion of connective tissue in the kidney. In contrast, Leach et al.[29,30] found no renal toxicity in rats repeatedly exposed for a period of 12 months to uranium dioxide dust (5 mg/m^3) (or in dogs or monkeys exposed for 5 years). Yet uranium concentrations in the kidney were as high as 1.1 μg U/g kidney wet weight in the rat (8.3 in the dog and 17.0 in the monkey), levels reported to cause acute renal toxicity (e.g., [25]). Thus the chronic effects of uranium exposure remain, for the most part, unresolved[8].

The threshold concentration of kidney uranium levels in man that results in kidney chemical toxicity is in dispute[8,26,52]. While the Nuclear Regulatory Commission has set the level at 3 μg/g kidney for renal damage in man, there is evidence from both human and animal reports that this level could be much lower. For example, chronically exposed uranium mill workers, whose kidney uranium levels probably did not exceed 1 μg U/g[54], showed mild renal dysfunction with increased urinary excretion of beta$_2$-microglobulin and various amino acids. In rats exposed subchronically to low doses (cumulative dose: 0.66 or 1.32 mg/kg) of uranyl fluoride, kidney uranium levels as low as 0.7 to 1.4 μg U/g wet kidney produced cellular and tubular necrosis of the proximal tubule, proteinuria, and enzymuria[9]. These changes in rat renal function, however, were temporary, with complete recovery within 35 days after exposure. These studies are important because they indicate that renal injury can occur at kidney uranium levels well below the 3.0μg U/g limit.

Neurological effects have been reported with uranium exposure. In uranium workers excreting up to 200 μg U/l in their urine, normal mental function was disrupted[24]. One case study linked the handling of a uranium bar and a subsequent increase in stool uranium with foot cramps, leg pain and abnormal gait[15]. With oral and subcutaneous administration of relatively high doses of uranyl acetate (210 mg/kg and 10 mg/kg, respectively), rats exhibited tremors[11]. The uranyl ion has been demonstrated to enhance muscle contraction with acute local concentrations of 200-400 μM[13,32]. At the neuromuscular junction in the mouse, multiple sites of action were identified, including increased duration of the muscle action potential, broadening of the compound nerve action potential, increased amplitude and quantal content of the endplate potential and increased frequency of the miniature endplate potentials[32]. These studies indicate that embedded DU fragments could lead to neural damage, affecting both motor and cognitive function. The CNS effects of uranium toxicity can result from secondary mechanisms since hormonal changes, electrolyte disruption and immune responses can all influence nervous system activity [47].

Local Tissue Response and Capsule Formation: Foreign bodies in tissue elicit an immune response that can result in encapsulation. Even when encapsulated, DU fragments provide a local, chronic source of alpha-radiation. Within 10-15 cells of the fragment, the dose rate is expected to be approximately 8.5 Gy/yr. This radiation could result in injury or damage to local muscle or nerve tissue (axonal injury, demyelination)[48,58]. In addition, capsule formation around a DU fragment in close proximity to a nerve could increase the risk of compression injury to those nerves.

Encapsulation could limit the chemical toxicity of the DU fragments by decreasing the rate of release of the metal, as has been observed with lead[35]. Encapsulation can also result in the formation of pseudocysts. Pseudocysts were formed that contained fluid with very high concentrations of soluble lead and insoluble lead dioxide particles[33,35] and with "black pigment...firmly adherent..." to portions of the inner wall of the capsule[33]. If these cysts should rupture, the rapid release of this fluid could cause period spikes in circulating lead levels and result in acute lead toxicity 5 to 40 years after the initial injury[33,35,59]. Similar type lesions may form around DU fragments. Intracapsular fluid may contain high concentrations of both soluble and insoluble DU. Tonry[55] demonstrated that DU disks formed both a soluble fraction and black insoluble particulates when emersed in simulated lung fluid. After a large fragment (approx. 20 mm) was removed from a U.S. soldier 17 months after he was wounded, the surgeon[28] noted that the fragment was encased in a fibrous capsule. When the capsule was breached, approximately 1-2 ml of a black fluid "gushed forth" from the cystic space.

DU can cause both local and systemic toxicity through a variety of mechanisms. Our study defines many

of the potential sites of pathology that can result from long-term exposure to DU fragments and will provide a rationale for treatment of our wounded soldiers. The first six months of the study established the doses of DU to be used in future experiments (aim 1). This dose ranging study determined the number of DU pellets required to obtain uranium levels in the range of 0.7 to 1.4 μg/g wet weight of kidney. This level of uranium has been reported to produce early signs of renal damage as measured by both biochemical and histopathological changes[9] and would define the high dose in our toxicological studies. The low dose was chosen to produce no measurable acute toxicity. Subsequent experiments use the established doses to evaluate neurotoxicity, nephrotoxicity and histopathology and determine uranium distribution for biokinetic modeling.

Neurotoxicity is assessed by (a) a battery of behavioral tests to assess functional consequences and (b) conduction velocity studies in motor nerves to uncover any peripheral neuropathies. Behavioral tests have frequently been employed to detect and characterize potential neurotoxic effects in rodents and have been used extensively in animal toxicity studies[44]. The neurobehavioral battery consists of (i) a functional observational battery (FOB), which is a series of tests designed to assess the neuromuscular, autonomic, and sensory integrity of the rat[12,36,37,39,40], (ii) an automated test of locomotor activity and (iii) the passive avoidance test used to evaluate memory. Electrophysiological experiments monitor nerve conduction velocity and integrity of the neuromuscular response. Nerve conduction velocity studies have been used clinically for many years to diagnose peripheral neuropathies and can even detect subclinical neuropathy induced by lead exposure[20,41,49].

Markers of renal function in the urine and plasma are used to assess nephrotoxicity. Altered creatinine clearance and proteinuria can indicate glomerular damage although tubular changes can also contribute. Increased urine content of enzymes such as lactate dehydrogenase (LDH) and N-acetyl-β-glucosaminidase (NAG) have been interpreted to reflect tubular damage[46]. In addition, appearance of glucose in the urine, can indicate alterations in tubule reabsorption. These markers have demonstrated sensitivity with acute uranium nephrotoxicity[8,9,31,63] and should indicate any toxicity that might result from long-term exposure to DU fragments.

Capsule formation and the sporadic release of pseudocyst fluid-contents can significantly influence the time course and concentration of uranium distributed through the body. The encapsulation process and pseudocyst formation is characterized at the time of euthanasia (1, 6, 12, 18 months after implantation), surrounding tissues are histologically examined and any capsular fluid is analyzed for its uranium content. In addition, tissues that are known to accumulate soluble uranium or uranium particulates (liver, bone, kidney, spleen)[19,27,29,30,61,62] are histologically evaluated.

Although the distribution of uranium in the rat has been characterized for a variety of routes of internalization (inhalation, ingestion, and parenteral administration of soluble compounds), this information is not available for embedded fragments. We are measuring uranium in urine, plasma, kidney, bone (tibia and skull), liver, spleen, brain, and skeletal muscle that is proximal and distal from the embedded pellets. Uranium is transported in plasma and urine and is stored in kidney and bone[19,27,61,62]. Uranium has been detected in the liver and spleen of animals[19,29,30] as well as in human subjects[23]. The skeletal muscle is being sampled to determine the local concentrations of uranium. The brain was chosen because of the paucity of data and the need to assess whether any neurological effects observed were due to the direct or indirect interaction of uranium in the body. These data will allow a rat biokinetic model for implanted DU fragments to be developed.

METHODS

Approach: This report describes the data obtained in the first two years of a three year study of 325 rats which will provide toxicity data for 3 DU doses (low, medium, high) at 4 time points (1, 6, 12, 18 months). Each rat is thoroughly evaluated for changes in behavior, peripheral nerve function, CNS excitability, renal

function and tissue histology including capsule formation. In addition, data on tissue uranium levels from a subgroup of rats are used to develop a biokinetic model to predict uranium distribution.

Rats are randomly assigned to 5 treatment groups: 1) rats implanted with low-dose DU, 2) rats implanted with medium-dose DU, 3) rats implanted with high-dose DU, 4) rats implanted with tantalum (Ta) to control for fragment implantation, and 5) a non-implanted sham-surgical control group. In the low-dose and medium-dose groups, Ta is substituted for a fraction of the DU pellets in order to keep the total number of implanted fragments constant. Half of the total number of pellets are implanted in each thigh.

Based on the variance of control data for neurological effects, a group size of 15 rats is necessary to see significant changes of 20% or greater at the $p<0.05$ level. Additional animals (20 rather than 15) have been implanted for the 18 month time point with the expectation of approximately 25% natural mortality[64-66]. This will provide 15 animals for analysis of neurological and biochemical endpoints in all groups at all 4 time points. Five of the rats in each experimental group provide tissue for uranium quantification. At the time of euthanasia, tissues from 7 animals per group at each time point will be assessed for uranium content and the remainder will be evaluated for histopathology.

Two-way analysis of variance is used to test statistical significance of any changes. Newman-Keuls test is used for multiple comparisons. In all analyses, statistical significance is accepted at the $p<0.05$ level.

Since this is a progress report, the total number of animals reported here do not represent the number of animals that will be included in the final analysis. Because of the staggered experimental schedule required for these protocols, the number of subjects for each endpoint will not be identical at the time of this report.

Subjects: Sprague-Dawley rats (8-10 weeks of age) are maintained in an AAALAC-accredited facility in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 86-23). Upon arrival, rats are quarantined and screened for diseases. Except during urine collection, all animals are housed in plastic microisolator rat cages with hardwood chips as bedding. Commercial rodent chow and water are provided *ad libitum*. Rats are on a 12-hr light/dark cycle.

Fragments: DU fragments, consisting of 99.25% DU and 0.75% titanium by weight, were obtained from Oak Ridge National Laboratories, Oak Ridge, TN. The uranium isotopes present is ^{238}U (99.75%), ^{235}U (0.20%) and trace levels of ^{234}U. This is the same DU alloy used in U.S. military munitions. Tantalum (Ta) fragments were obtained from Alfa Products, Ward Hill, MA. Ta was chosen as the control substance because it is a biologically inert metal[22] with a similar mass to uranium and is frequently used in human prostheses[21,53]. Each fragment (both DU and Ta) is approximately 1 mm diameter x 2 mm long.

Experimental groups: Rats were divided into five experimental groups: (1) non-surgical controls (2) Ta controls (3) high dose of DU (4) medium dose of DU and (5) low dose of DU. The preliminary dose ranging study determined that 20 DU pellets produced urine uranium levels of 262±99 μg U/l and kidney levels of 1.22 ± .31 μg/g after two weeks of exposure. This dose was chosen as the high dose because it was well tolerated by the rats but expected to produce kidney toxicity. Consequently all surgically implanted animals have a total of 20 pellets of either Ta or DU or a mixture. Low dose DU rats are implanted with 4 DU pellets and 16 Ta pellets. Medium dose DU rats are implanted with 10 DU and 10 Ta pellets.

Surgery: The DU and Ta pellets are cleaned and chemically sterilized prior to implantation. The pellets are immersed in industrial detergent, rinsed in absolute alcohol, soaked in 50% nitric acid solution for 3 min and then rinsed with water. This procedure completely removes the oxide formation on the surface of the DU pellet[55]. Anesthesia is induced with ketamine hydrochloride (80 mg/kg) in combination with xylazine hydrochloride (4 mg/kg), given i.p.

Fragments are implanted within the gastrocnemius muscle spaced approximately 8-10 mm apart on the lateral side of each leg. The surgical sites are shaved and cleansed with betadine, a topical disinfectant, prior to surgery. Scalpel incisions are made through the skin and pellets are inserted into the muscle with a trochar (16 gauge needle with plunger). Incisions are closed with absorbable sutures and surgical cement.

Rats are closely monitored following surgery until they are ambulatory and an analgesic (Demerol, 10 mg/kg, i.m.) is administered if needed. A veterinarian regularly examines the surgery sites for signs of inflammation, infection and local DU toxicity.

Behavioral neurotoxicity: The functional observational battery (FOB) consists of behavioral evaluations (home-cage, handling and manipulative) and several physiological measures. The parameters to be recorded are listed below and grouped according to the following functional domains: 1) Autonomic: lacrimation, salivation, palpebral closure, piloerection, defecation, urination, 2) Sensorimotor reactivity: tail pinch response, tactile response, click response, approach response; 3) Neuromuscular: gait, foot splay, forelimb and hindlimb grip strength, righting reflex, and 4) CNS Excitability: arousal, posture, ease of removal from cage, handling reactivity, convulsions, and locomotor activity.

The observer is blind as to the identity of each group. The behavioral battery commences with brief home cage observations during which time the observer describes the posture, and the existence of tremors or convulsions, and palpebral closure. The rats are then removed from their cage and rated for ease of removing and handling. While handling the rat, presence of piloerection and the degree of lacrimation and salivation are observed. The animals are then placed in an open-field with a perimeter barrier on clean absorbent white paper for 3 min. The number of rears, the gait, level of alertness, stereotypy (repetitive movements e.g., head weaving), unusual behaviors (e.g., writhing), and the number of fecal boli and urine pools are recorded.

Sensorimotor responses also are determined and include: approach response to a blunt probe, touch on the rump (tactile response), click response (auditory response), and pinch on the tail using forceps. Next, neuromuscular responses are determined and include: righting reflex, forelimb and hindlimb grip strength using digital strain gauges[37], and landing foot splay[12]. The animals are weighed and rectal temperature determined using a digital thermometer. The FOB is conducted during the light portion of the light-dark cycle. Details of the FOB tests can be found in Moser et al.[40] and McDaniel and Moser[36].

Approximately, 1 hr after the FOB, the rats are monitored for horizontal and vertical locomotor behavior. Motor activity is recorded for 1 hr using automated photocell activity cages (Digiscan Analyzer, Omnitech Electronics, Columbus, OH). On the day following the FOB and motor activity tests, animals are trained on a passive avoidance test. This test is used to determine whether DU affects memory function. The tests are conducted in a passive avoidance apparatus (San Diego Instruments, San Diego, CA) that consists of two chambers (one lighted, one darkened) separated by a sliding door. The animal receives a training trial during which time it is initially placed into the lighted chamber. The natural tendency is for the rat to enter the darkened chamber. When it does, it receives a mild foot shock. During this acquisition phase, the rats are tested for eight trials or until criterion is met. The criterion is two consecutive trials during which the rat does not cross into the darkened chamber. Each trial is 3 min in duration with a 1 min intertrial interval. Seventy-two hours later the rat is placed into the lighted chamber and retested. A comparison is made with the initial training session to see if memory of the task has been retained.

Conduction velocities: One week following the behavioral testing, the rats are evaluated electrophysiologically. Rats are anesthetized with ketamine (80 mg/kg) with xylazine hydrochloride (4 mg/kg) i.m. (supplemented as necessary). The right sciatic nerve is exposed and bipolar stimulating electrodes are positioned along the nerve in the thigh close to the sciatic notch and in a second location close to the knee. A recording electrode is inserted into the medial gastrocnemius muscle to monitor the compound muscle action potential. Nerve temperature is monitored and maintained near 37 °C with a heat lamp. Nerves are stimulated at a frequency of 0.2 Hz. Stimulus intensity is varied between approximately 10 and 100 V (0.1 ms duration) to determine the input-output relationship and the supramaximal stimulation parameters to use. Five muscle responses are averaged and the latency, duration and amplitude of the potentials are measured. Conduction velocities are calculated by dividing the distance between the stimulating electrodes by the average latency difference between the time of onset of the compound muscle action potentials.

Duration of the muscle action potential reflects the synchrony of discharge. In general, the distal stimulating electrode will produce a faster, larger response than the proximal electrode. Greater dispersion

and greater decrease in amplitude than normal would suggest nerve damage. For example, demyelinating disorders cause dispersion of the muscle action potential by slowing the nerve conduction velocities[5,50]. If dispersion occurs over a short segment, compression neuropathy may be indicated[5].

All stimulation and recording are controlled by a 486 PC using standard electrophysiological software (Axon Instruments). Data are analyzed with routines written in AxoBasic (Axon Instruments) and statistical analysis is done with RS/1 (BBN Software Products) routines. Two-way analysis of variance (for time and dose) is used to compare differences among the experimental groups.

Hippocampal slice electrophysiology: At the termination of the conduction velocity experiment, the rat is euthanized by decapitation. The brain is quickly removed from the skull and submerged in iced oxygenated artificial cerebrospinal fluid (ACSF). Both hippocampi are dissected out and sliced on a McIlwain tissue chopper (425 μm thick). Tissue is incubated at room temperature in oxygenated ACSF (see below) for 1 hr to allow recovery from the slicing procedure. During this interval, tissues are isolated from the rats for analysis of histopathology and DU content.

A single slice of rat hippocampus is then be placed in a submerged slice chamber and perfused at a rate of 1-2 ml/min with warmed (30 °C) oxygenated ACSF. ACSF has the following composition (in mM) 124 NaCl, 3 KCl, 2.4 $CaCl_2$, 1.3 $MgSO_4$, 1.24 KH_2PO_4, 10 glucose, 26 $NaHCO_3$, pH 7.4, equilibrated with 95% O_2/5% CO_2. Extracellular recordings are obtained with glass microelectrodes filled with 2 M NaCl placed in s. radiatum and s. pyramidale of field CA1 to record the population synaptic potential (pPSP) and the population spike (PS) respectively. A stainless steel, concentric, bipolar stimulating electrode is positioned in s. radiatum of field CA1 to activate afferents. Constant current stimuli (0.1 - 1.5 mA, 300μsec) are applied at a frequency of 0.2 Hz. Except when generating input/output (I/O) curves, the stimulus current is held constant at an amplitude that elicits approximately 30% maximal response.

To obtain I/O curves, stimulus intensity is varied from approximately 0.1 to 1.5 mA in 13 steps. Three responses at each current step are recorded and averaged. I/O curves are generated following a 30-min equilibration period. The three I/O curves (stimulus vs PS, stimulus vs pPSP, pPSP vs PS) are analyzed with the data analysis software RS1 (BBN Software Products, Cambridge MA). The responses at each stimulus intensity are averaged for all experiments at each time point. A sigmoid curve is computer fit to the points. Differences between curves are tested for significance by comparing the residual sum of squares for the curve fit to the data of each experimental condition with the residual sum of squares for the curve fit to all the data. Significance is accepted at $p<0.05$.

Sample collection: Following behavioral testing, blood and urine samples are obtained from all rats for analysis of renal function. To safely collect the blood samples, rats are immobilized by placing them in a Plexiglas restrainer. During each collection, 0.3-0.5 ml of blood is obtained from the tail vein using a 22-gauge needle. The blood is then centrifuged for 5 min at 3,000 X g. The serum is analyzed for uranium levels and/or for biochemical indices of renal function. Serum is stored at -70 °C until ready for analysis.

Urine samples are collected by housing the rats in individual metabolism cages (23.5 cm diameter X 12 cm high) where they have continuous access to food and water. However, since these housing procedures have been shown to induce stress and thus increase the toxicity of uranium[4], the rats are acclimated to the metabolic cages for 5 days before the study begins. The metabolic cages are disinfected and decontaminated between each animal use. The 24-hr urine collection sample is obtained from each rat and the volume recorded (10-20 ml). Urine collection at 4 °C is unnecessary since enzyme activity has been shown to be stable at room temperature for up to 24 hours[63]. After collection, urine is filtered to remove any debris and stored in plastic containers at 4°C until analyzed (less than one week).

Evaluation of renal function: Measurement of urine volume and osmolarity, urine levels of NAG, LDH, glucose, total protein, creatinine and blood levels of glucose, urea and creatinine are used as indicators of renal function. In addition, since weight loss may be indicative of nephrotoxicity, all the rats are weighed weekly throughout the study. Osmolarity of the urine is measured with a vapor pressure osmometer (Model 5100B, Wescor, Inc.). A Kodak Ektachem 700 Analyzer is used to determine plasma and urine

levels of creatinine, glucose and urea. Total urine protein is measured with a dye-binding assay (Coomassie Blue, BioRad) sensitive down to 1 μg. The activity of NAG is measured by the methods of Tucker et al.[56] using 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide as the fluorescent substrate (excitation wavelength=356 nm; emission wavelength=446 nm). The dilution of the urine for this assay eliminates the effects of any inhibitors present[56]. For LDH measurements, 1 ml of urine is dialyzed for 4 hr at 4 °C with 1 liter of deionized water. LDH is quantitated with a colorimetric assay that measures a reaction product which is proportionate to LDH activity (Oxford Biomedical Research Inc). Only 50-100 μl of fluid (urine or plasma) are required for each of these assays.

Although, urine volume and osmolarity can vary greatly with fluid intake, these measures provide physical indicators of renal function. For example, acute kidney failure drastically decreases urine volume, while moderate renal toxicity can increase urine output, as is seen with uranium exposure (e.g., [11]). Osmolarity can reflect the ability of the kidney to concentrate (or dilute) the urine. Plasma urea also changes with renal insufficiency. Since the rate of urea formation is proportionate to the rate of protein metabolism, other factors such as hepatic injury or altered protein intake can affect the measured urea in plasma. A small concentration of protein is normally present in the urine. Increases in total urine protein could result either from glomerular leakage or failure of tubule reabsorption. Urinary enzymes are sensitive, non-invasive markers of toxicity primarily in the kidney tubules[46]. NAG is a lysosomal enzyme found in proximal renal tubule cells. LDH is a cytosolic enzyme of the tubular epithelium.

Creatinine clearance is a commonly used measure of glomerular filtration rate in the rat despite a significant but constant tubular secretion. The use of an intrinsic metabolite has an obvious advantage over inulin or mannitol which (although not secreted) must be infused. Interpretation must be cautious since tubular injury with uranium could cause an underestimate of the glomerular filtration rate regardless of the marker used[8]. Creatinine clearance (C_c) is calculated from the equation: $C_c=U_c*V_u/P_c$ where U_c and P_c are the creatinine concentrations in urine and plasma, respectively, and V_u is the rate of urine production (ml/min).

Appearance of glucose in the urine occurs when the tubule reabsorption maximum from the filtrate is exceeded. This can occur with hyperglycemia or with a decrease in tubular reabsorption capacity. Measurement of both urine and plasma glucose help to distinguish between these two possibilities. Changes in reabsorption is reflected in the calculated fractional excretion (FE): $FE = (U_g/P_g))(U_c/P_c)$; where U_g and P_g are the glucose concentrations in urine and plasma, respectively.

The proposed assays provide a broad spectrum of measures of kidney toxicity. Many of these substances have been shown to be very sensitive in acute uranium toxicity[8,31]. Glucose is one of the most sensitive indicators[8,9] showing increased urine glucose, without concurrent increases in plasma. LDH and to a lesser extent NAG increase following uranium exposure[8,31]. A transient increase in urine volume and the appearance of protein in the urine also occur with acute uranium toxicity[31]. These measures are used together as indicators of kidney toxicity and carefully interpreted and correlated with histopathology. Two-way ANOVA is used to test the statistical significance of any changes.

Histopathology. Immediately following euthanasia on the day of electrophysiological analysis, tissue samples from bone (tibia, skull), hippocampus, sciatic nerve, kidney, liver, spleen and fragment capsule with associated skeletal muscle is obtained for histological examination or uranium measurement. Based on the literature, these are the most likely tissues to show increased levels of uranium[19,27,29,30,61,62]. Standard procedures for handling biologic specimens are used in the preparation of the samples. Tissues are perfused, embedded, mounted and stained with hematoxylin and eosin stain (H & E)[34]. Specialized stains are used to demonstrate specific lesions or further delineate lesions not well defined by the H & E stain. For example, silver stains are used on neural tissue to delineate nerve fiber disruption or degeneration[34].

The pathologist evaluating the tissue is blind to the experimental group from which the tissue was obtained. The pathologist generates a 0 to 4 scoring system to evaluate the degree of microscopic changes observed; where 0=no change, 1=minimal change, 2=mild change, 3=moderate change, and 4=marked or severe change. All tissue changes observed in the rats implanted with DU are contrasted and compared to the

identical tissues taken from the controls. If there are significant changes noted in a particular system, for example the renal system, a detailed statement of criteria for 0-4 scores is stated by the pathologist at the time of interpretation.

Uranium measurement Tissue samples are frozen and shipped by overnight courier on dry ice to Battelle, Pacific Northwest Laboratories for analysis of uranium content. The samples are stored at -70° C until the wet ashing procedure. Wet ashing consists of 12 cycles of treatment of the samples (over 3 days) with 2 ml of 16 N nitric acid followed by several hours of heating, brief cooling, addition of 0.5 ml of 30% hydrogen peroxide and reduction of the volume to approximately 0.5 ml. After this, samples are heated to dryness, dissolved in 2 ml of 4 M nitric acid with warming and filtered through 0.45 μm syringe filter units. For analysis, 0.5 ml of sample or identically handled standards are dissolved in 2 ml of Uraplex reagent. The samples are analyzed with a Kinetic Phosphorescence Analyzer (KPA-11, Chemchek Instruments Inc, Richland WA). Background measurements are made using 4 M nitric acid. Calibration curves are established prior to sample analysis. Measurements include analysis of relative standard deviations and correlation coefficients of the luminescence decay curve.

RESULTS

Because of the staggered experimental schedule required for complete analysis of the numerous experimental endpoints, all subjects have not been evaluated to date. Sample sizes, therefore, will vary.

Uranium distribution: Tissues and fluids from five rats of each of the five experimental groups for the 1-day, 30-day and 6-month time points have been analyzed for uranium content by Battelle Pacific Northwest Laboratories. Urine was not collected from the rats at the 1-day time point because the complications introduced by the surgery and the procedures for the collection of the fluid. At all time points uranium distributed primarily to bone and kidney in a dose-dependent manner (Figures 1, 2, 3). Even at day one, uranium was present in these tissues (Figure 1). By day 30, the levels in kidney and bone had increased and uranium was evident in the urine (Figure 2). In addition, there was evidence of uranium distributing to the spleen and to the brain of the high dose DU animals. At 6 months, the levels in tibia, kidney, urine and brain had continued to rise while levels in the skull had plateaued out (Figures 3-9). Distribution to the spleen in the high dose DU animal had leveled out but for the low and medium doses continued to rise (Figure 8). Levels in the liver were comparable to controls at the 30-day time point but increased slightly at 6 months (data not shown). Muscle levels were, in general, quite variable. Some of the muscle samples that were in close proximity to the DU pellets showed exceptionally high levels of uranium. It is our belief that these high levels resulted directly from fragments of the implanted pellets in the analyzed samples. This "contamination" could have occurred during the removal of the pellets at time of necropsy or might have happened by flaking and redistribution *in vivo*. Further analyses are expected to clarify this issue.

Histopathology: Tissues have been excised and fixed for histopathological analysis. These tissues (bone: tibia and skull, kidney, spleen, liver, brain, and muscle: proximal and distal). No histological evidence of toxicity has been observed. Figure 10 shows the renal cortex at 6 months in one high dose DU rat and a Ta control. There is no evidence of cellular necrosis, inflammation or fibrosis. During excision of the pellets it was observed that the DU pellets but not the Ta pellets were associated with adherent tissue. Even in the 6-month animals, a capsule had not fully formed around the pellets and dark fluids were not observed near the fragments.

Nephrotoxicity: The urine and serum samples have been analyzed for biochemical markers of kidney toxicity. Osmolarity, 24-hour volume, pH and urine levels of glucose, protein, NAG, LDH, urea nitrogen, serum glucose and serum urea nitrogen were not significantly altered at either the 30-day or 6-month time points. The data for urine osmolarity, glucose, protein and NAG are shown in Figures 11-14. Creatinine clearance was not significantly different among the experimental groups (Figure 15). Fractional excretion (FE) of glucose (glucose clearance/creatinine clearance) was similarly not significantly affected by the experimental procedures with all groups showing an FE between 0.0025 and 0.0038 at the 1-month time point and between 0.0008 and 0.0015 at the 6-month time point. The differences were not statistically significant ($p>0.2$).

Neurotoxicity: Animals were evaluated for body weight and for changes in the functional observation battery (FOB), locomotor activity, and passive avoidance learning. The rats were weighed weekly and all steadily gained weight. Animals in the high DU dose group have shown significant differences in the body weight compared to Ta controls at a number of time points (Figure 16) ($p<0.05$). Although not statistically significant, the medium-dose DU animals show the same trend toward lower body weights than the control animals.

The FOB did not reveal any significant differences among the experimental groups. No significant differences were observed in body temperature at 30 days or at 6 months. Sensorimotor, neuromotor and autonomic responses as well as locomotor activity showed no significant differences across the experimental groups at either time point. As expected, in all groups the initial locomotor activity was high when the animals were first placed in the activity monitors because of exploratory behavior which subsided over time

(Figure 17). Grip strength of the hind- and forelimbs was not significantly altered by DU exposure (Figure 18). Conduction velocity measurements from the nerves of the hindlimb also did not reveal any differences among the experimental groups at either time point (Figure 19).

Passive avoidance was used as a measure of learning ability. Any rats that failed to cross to the darkened compartment were dropped from the study. As a consequence the number of subjects reported for this endpoint is less than the number of animals in the study. For both the 30-day and the 6-month time points, there were no significant differences among the five experimental groups for their performance on the passive avoidance test (N=7-10/group at 30 days; N=4-9/group at 6 months). All animals learned to avoid the mild foot shock within 2-3 trials. The latency to initial crossover was also not significantly different among groups (Figure 20).

Because of the observed distribution of uranium to the brain only in the high DU animals at 30-days, hippocampal electrophysiology was assessed at 6 months in the Ta controls and 20-DU pellet rats. The population spike in the hippocampus of the DU implanted animals was significantly smaller than that in the controls (Figure 21). In contrast the differences in the size of the synaptic potentials were not statistically significant (data not shown). The input-output relationship reflecting the ability of the synaptic potential to elicit the population spike indicated that in the high-dose DU animals this process was significantly impaired ($p<0.05$).

Miscellaneous observations: During the year since all of the animals have been implanted with pellets, we have observed several pathological conditions that have resulted in the removal of the animal from the study as recommended by our veterinary staff.

Five rats developed problems with their teeth. Their upper teeth broke off and the lower teeth began to grow abnormally. All of these rats had DU pellets implanted (one low dose, two medium dose and two high dose). These animals had difficulty eating, showed weight loss and were euthanized.

Three rats (1 low DU dose, 1 medium DU dose, 1 high DU dose) were euthanized because they exhibited substantial weight loss accompanied by gasping or coughing. On necropsy they were found to have gas-filled intestines. Two animals (1 Ta control, 1 medium dose DU) were euthanized when they developed skin sores that failed to heal with antibiotic treatment.

Five additional animals have died or were euthanized when observed moribund. In three of these (2 low DU dose, 1 Ta control) no abnormal pathology was observed. The fourth rat (non surgical control) had an enlarged liver and lymphosarcoma was suspected. Necropsy of the fifth rat (non surgical control) revealed bladder stones.

CONCLUSIONS

The data presented here describe the distribution of uranium from intramuscularly embedded pellets to body tissues over six months and characterize the physiological and behavioral consequences of this exposure. The most striking findings of this study to date are 1) the absence of measurable nephrotoxicity despite very high levels of uranium in the kidney and urine and 2) the distribution of uranium to the brain and the changes in neuronal excitability in the hippocampus.

Uranium levels in the kidney of the experimental animals implanted with DU exceed the levels considered to be toxic in both animal models and man. The medium and high dose groups averaged kidney levels greater than 3 μg/g, the level set by the Nuclear Regulatory Commission for renal damage. At six months, the high, medium and low doses of DU pellets resulted in 6.9±1.7, 4.7±0.8 and 2.3±0.6 μg/g kidney, respectively. Examination of the time course of the uranium levels in kidney suggests that these values may continue to rise. Urine levels of uranium in the high and medium dose groups (674±156 and 243±52 μg/l respectively) exceed the levels found in Desert Storm veterans (Keogh, personal communication) while the low dose group (46±13 μg/l) showed levels comparable to that population. As with the kidney levels of uranium, the urine levels continue to rise over time. Despite these high levels, our data do not demonstrate any signs of nephrotoxicity. Chemical form, route of administration, and the dose of uranium exposure can all affect the toxicological consequences and distribution of uranium. It is possible that chronic exposure to uranium allows tolerance to higher concentrations of the metal. The studies of Leach et al.[29,30] demonstrate an absence of renal toxicity in rats following chronic inhalation exposure to uranium dioxide producing kidney levels up to 1.1 μg/g. In contrast, Diamond et al.[9] observed acute, but reversible, renal toxicity in rats at levels as low as 0.7 μg/g following i.v. injection of uranyl fluoride. The absence of effects in our present study does not preclude the possibility that with longer exposures to the uranium, toxicity will develop.

At the 30-day time point uranium was observed to distribute to the brain in the high DU dose animals. This was in agreement with the literature[45] in which uranium did not accumulate in the brain at the lower doses of DU. At 6 months, however, substantial amounts of uranium are accumulating in the central nervous system. Although behavioral measures did not reveal any adverse effects of this uranium, electrophysiological assessment of hippocampal neuronal activity, demonstrated that excitability was impaired. It is possible that the electrophysiological changes are too subtle to produce a behavioral manifestation. Alternatively, it is possible that the behavioral tests we have performed are not sufficiently sensitive to reveal an existing cognitive deficit. It also needs to be emphasized that only the high dose DU animals were assessed and these animals had levels of uranium in their urine that significantly exceeded the levels found in any of the veterans. Future studies will determine the electrophysiological correlates in all of the dose groups. In addition, it will important to assess the process of long-term potentiation, a physiological correlate of memory and learning in the brain slice preparation.

Bone, like kidney, is well accepted as a primary reservoir of uranium. As with kidney, uranium appears to be continuing to accumulate in the tibia of the DU embedded rats. In contrast to the marrow bone, skull levels are high but after 30 days appear to have saturated in their uranium concentration. This could be the difference between marrow and non-marrow containing bone. Perhaps more likely, the difference could be due to the continued growth of the tibia during this time period and the much slowed growth of the skull. If uranium is deposited with bone growth, we would predict that over the next 6 months (i.e., by the 1-year time point) levels in the tibia will also begin to saturate. Future studies should resolve this issue.

Other organs accumulate the uranium to varying degrees. At thirty days, concentrations in the liver were not above background while concentrations in the spleen and muscle were significantly higher. At 6 months levels in the liver had become statistically significant but were still exceeded by levels in the spleen. Muscle levels raise the possibility that neuromuscular deficits will develop through heavy metal effects.

Spleen levels cause concern that immunological consequences could arise. Future studies are planned to address this possibility.

These studies demonstrate the potential health hazards associated with exposure to depleted uranium shrapnel. The data suggest that chronic exposure to the uranium may not be as toxic to the kidneys as had been anticipated from acute exposure studies. In contrast, the distribution of uranium to the brain and the electrophysiological changes that result raise concerns about cognitive deficits that may increase over time. Furthermore, the distribution of uranium to the spleen may suggest potential immunological effects. Since the use of depleted uranium armaments is expanding, it is increasingly important to be aware of health risks associated with exposure in order to formulate an appropriate protocol for handling casualties with DU shrapnel.

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Figure 1

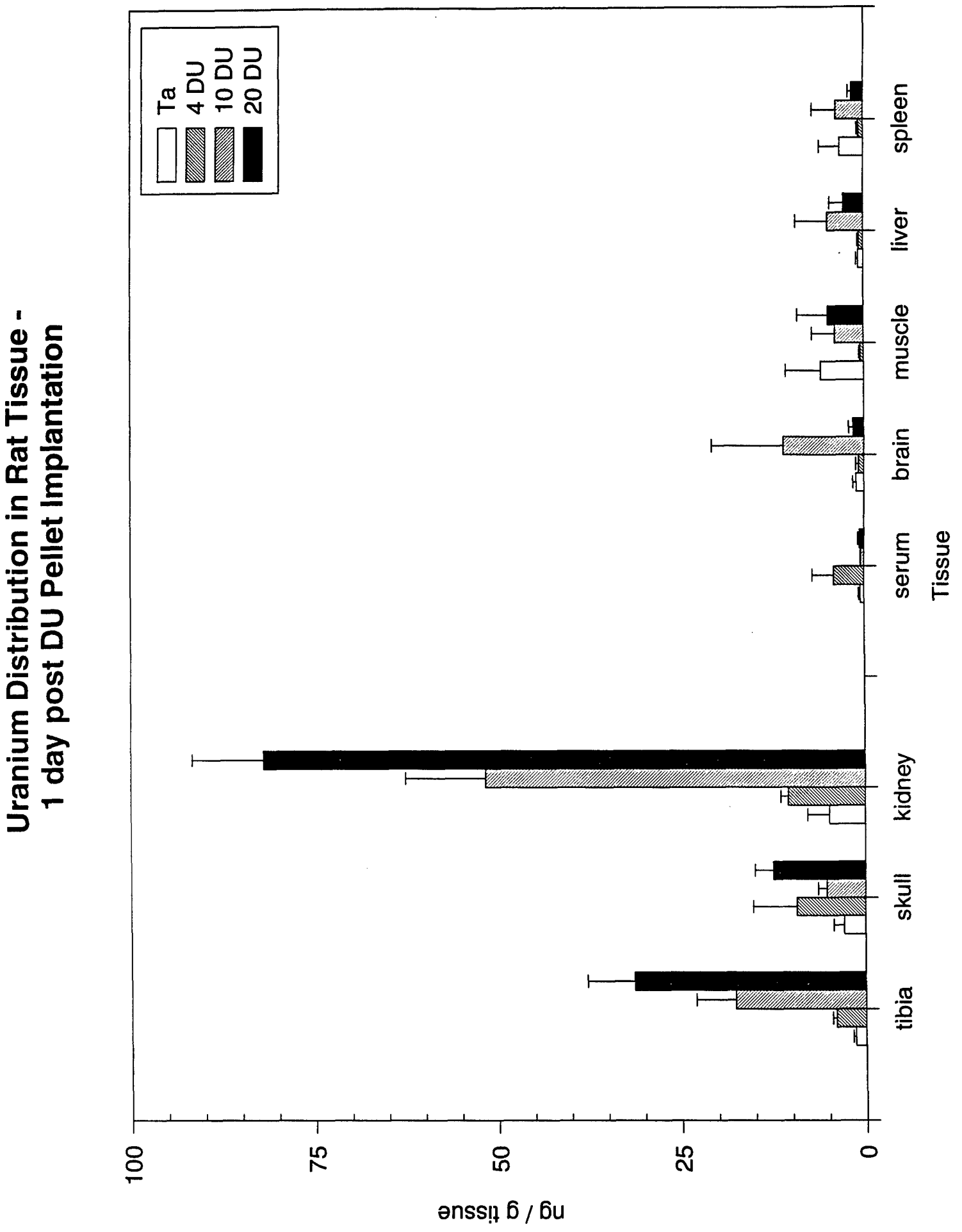

Uranium Distribution in Rat Tissue -
1 day post DU Pellet Implantation
Ta
4 DU
10 DU
20 DU
ng / g tissue
100
75
50
25
0
tibia
skull
kidney
serum
brain
muscle
liver
spleen
Tissue

Figure 2

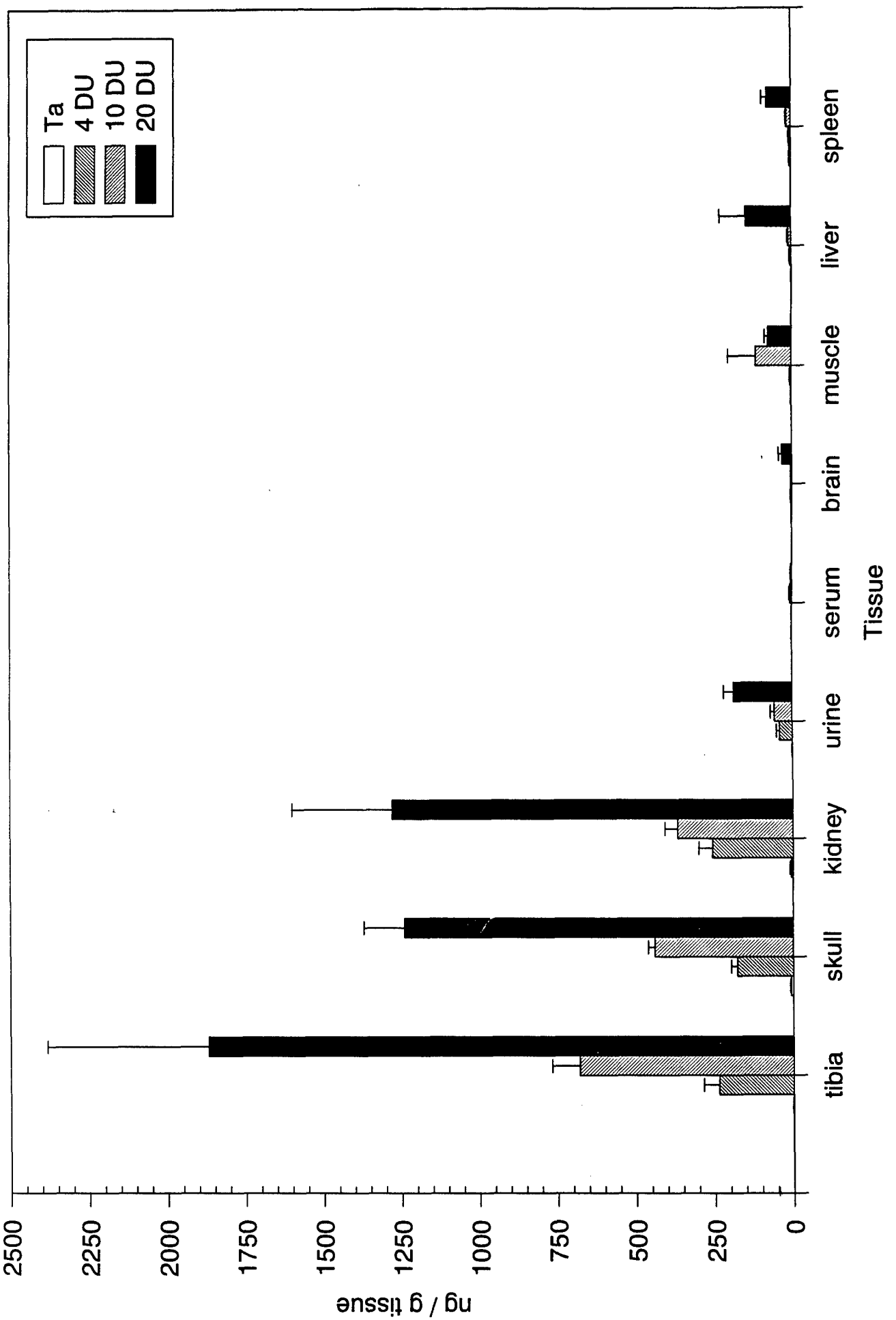

Uranium Distribution in Rat Tissue -
30 days post DU Pellet Implantation
Ta
4 DU
10 DU
20 DU
ng / g tissue
2500
2250
2000
1750
1500
1250
1000
750
500
250
0
tibia
skull
kidney
urine
serum
brain
muscle
liver
spleen
Tissue

Figure 3

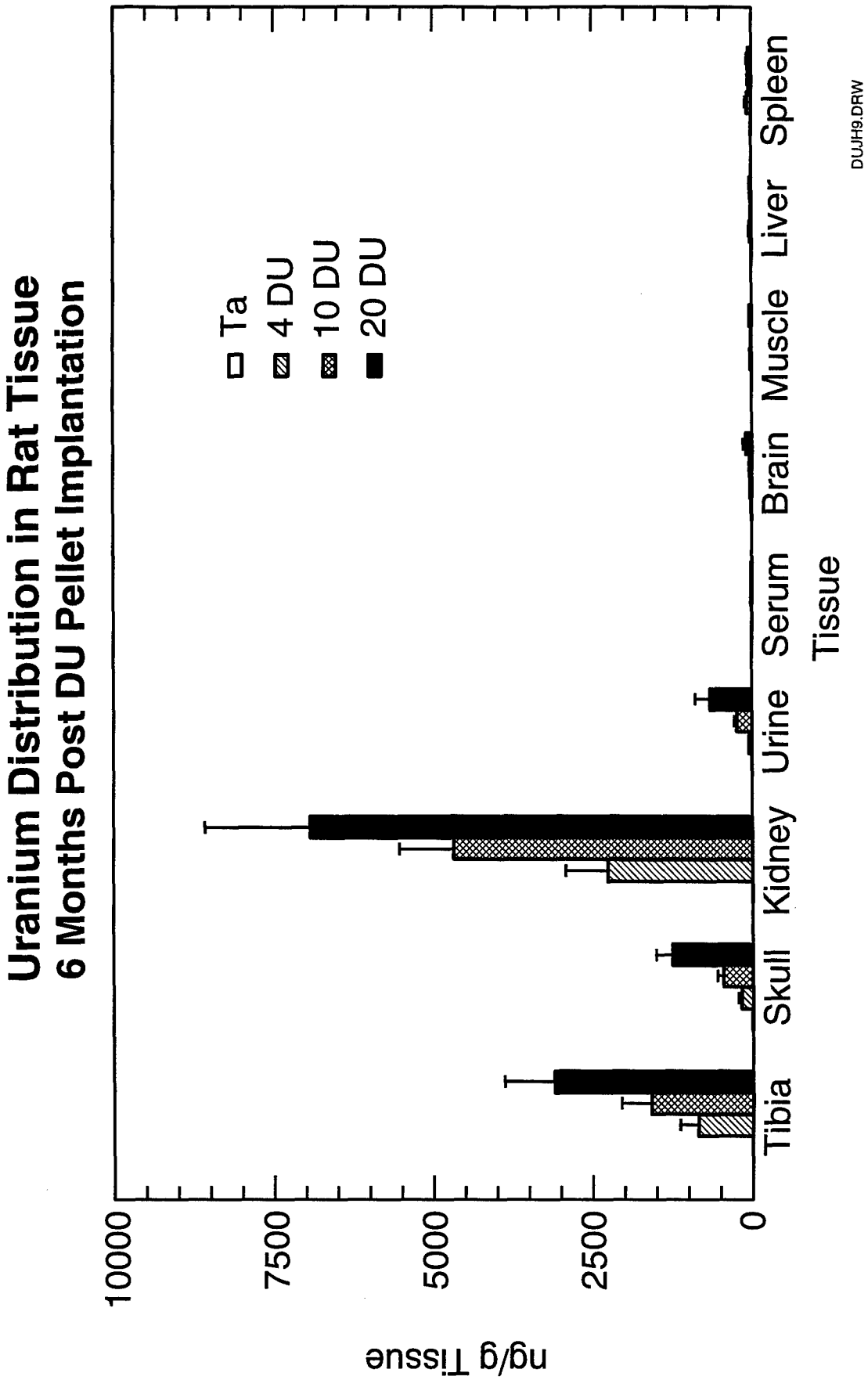

Uranium Distribution in Rat Tissue
6 Months Post DU Pellet Implantation
Ta
4 DU
10 DU
20 DU
10000
7500
5000
2500
0
ng/g Tissue
Tibia
Skull
Kidney
Urine
Serum
Brain
Muscle
Liver
Spleen
Tissue
DUJH9.DRW

Figure 4

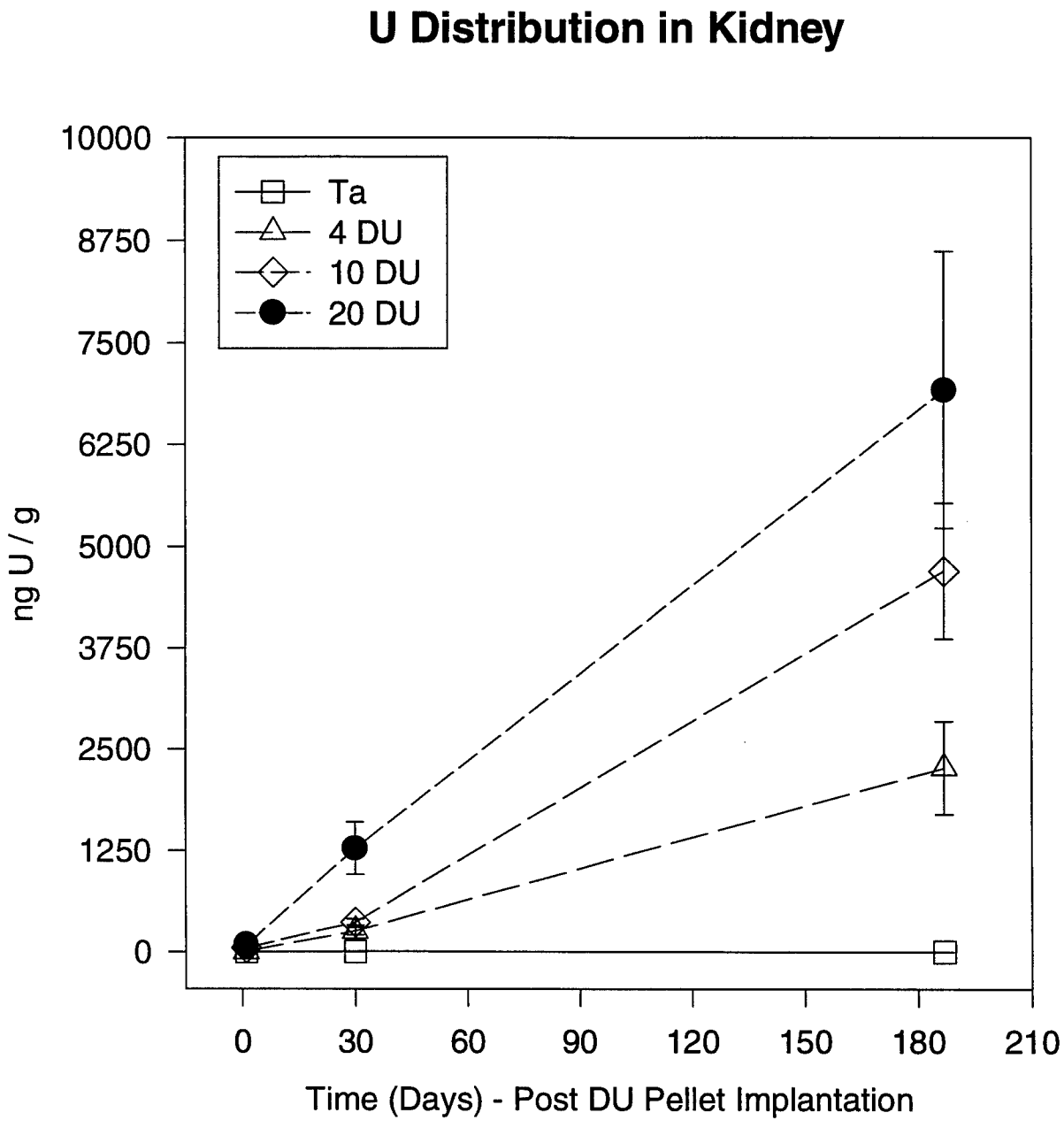

U Distribution in Kidney
10000
8750
7500
6250
5000
3750
2500
1250
0
ng U / g
Ta
4 DU
10 DU
20 DU
0
30
60
90
120
150
180
210
Time (Days) - Post DU Pellet Implantation

Figure 5

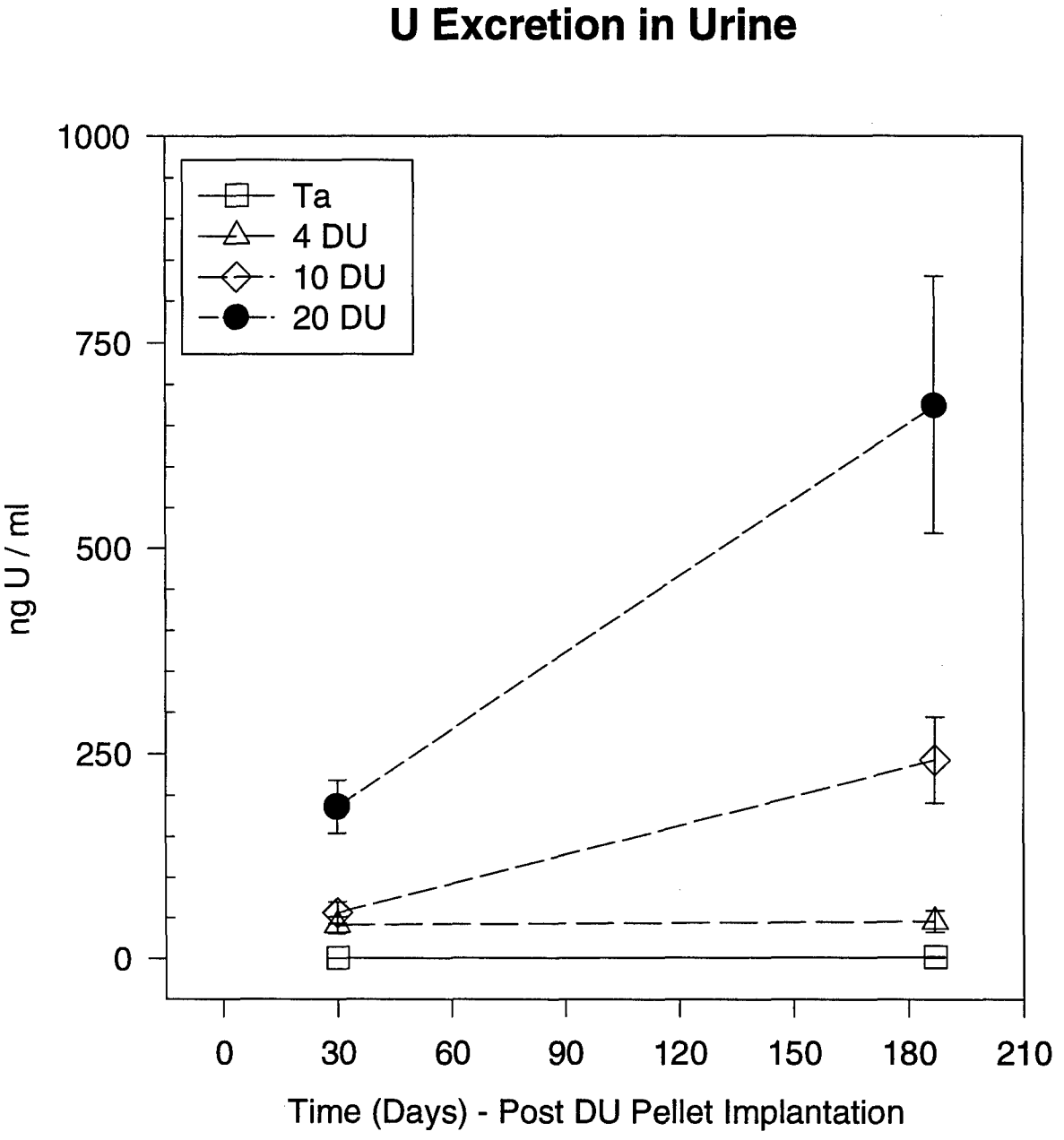

U Excretion in Urine
1000
750
500
250
0
ng U / ml
Ta
4 DU
10 DU
20 DU
0
30
60
90
120
150
180
210
Time (Days) - Post DU Pellet Implantation

Figure 6

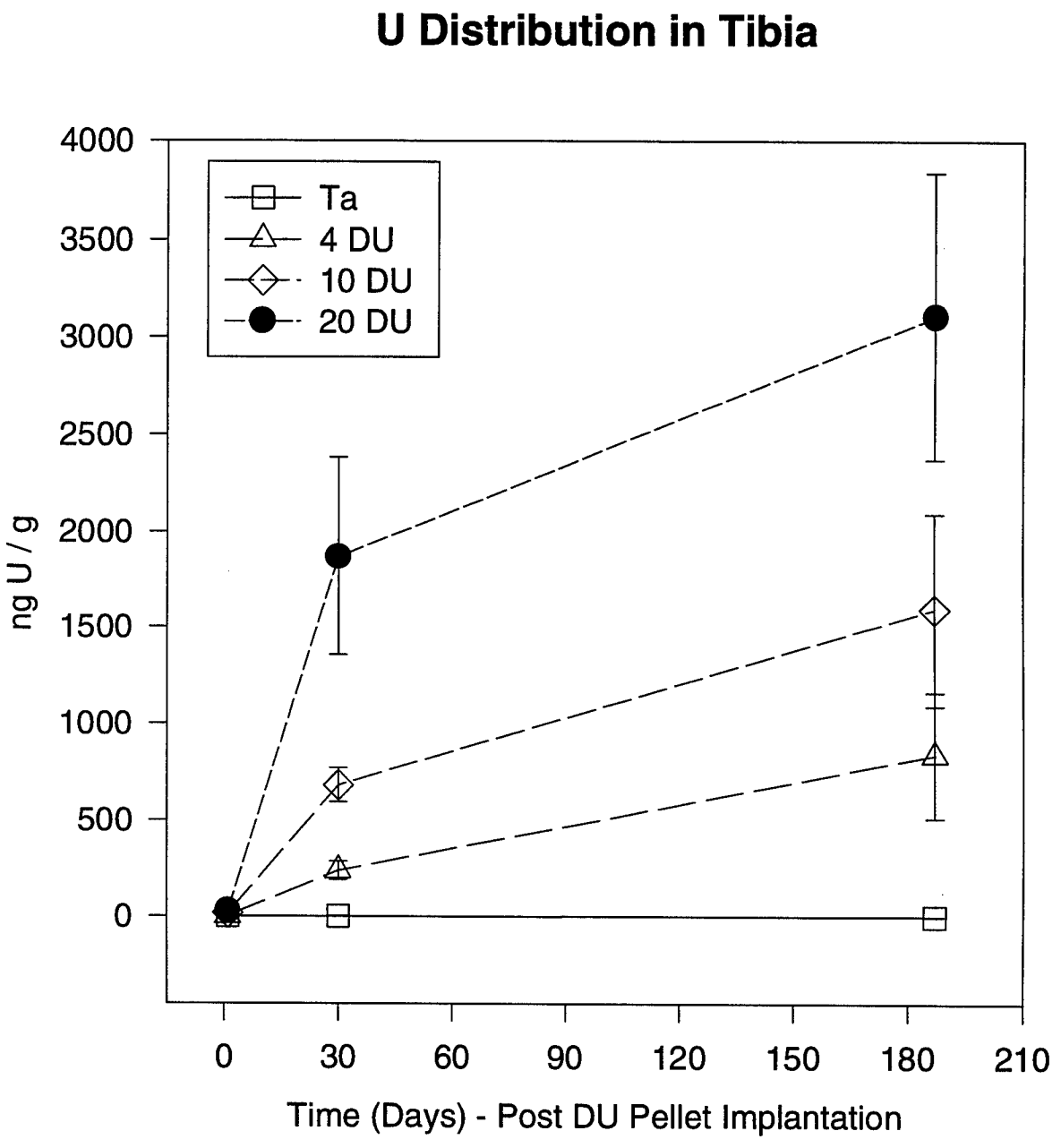

U Distribution in Tibia
4000
3500
3000
2500
2000
1500
1000
500
0
ng U / g
Ta
4 DU
10 DU
20 DU
0
30
60
90
120
150
180
210
Time (Days) - Post DU Pellet Implantation

Figure 7

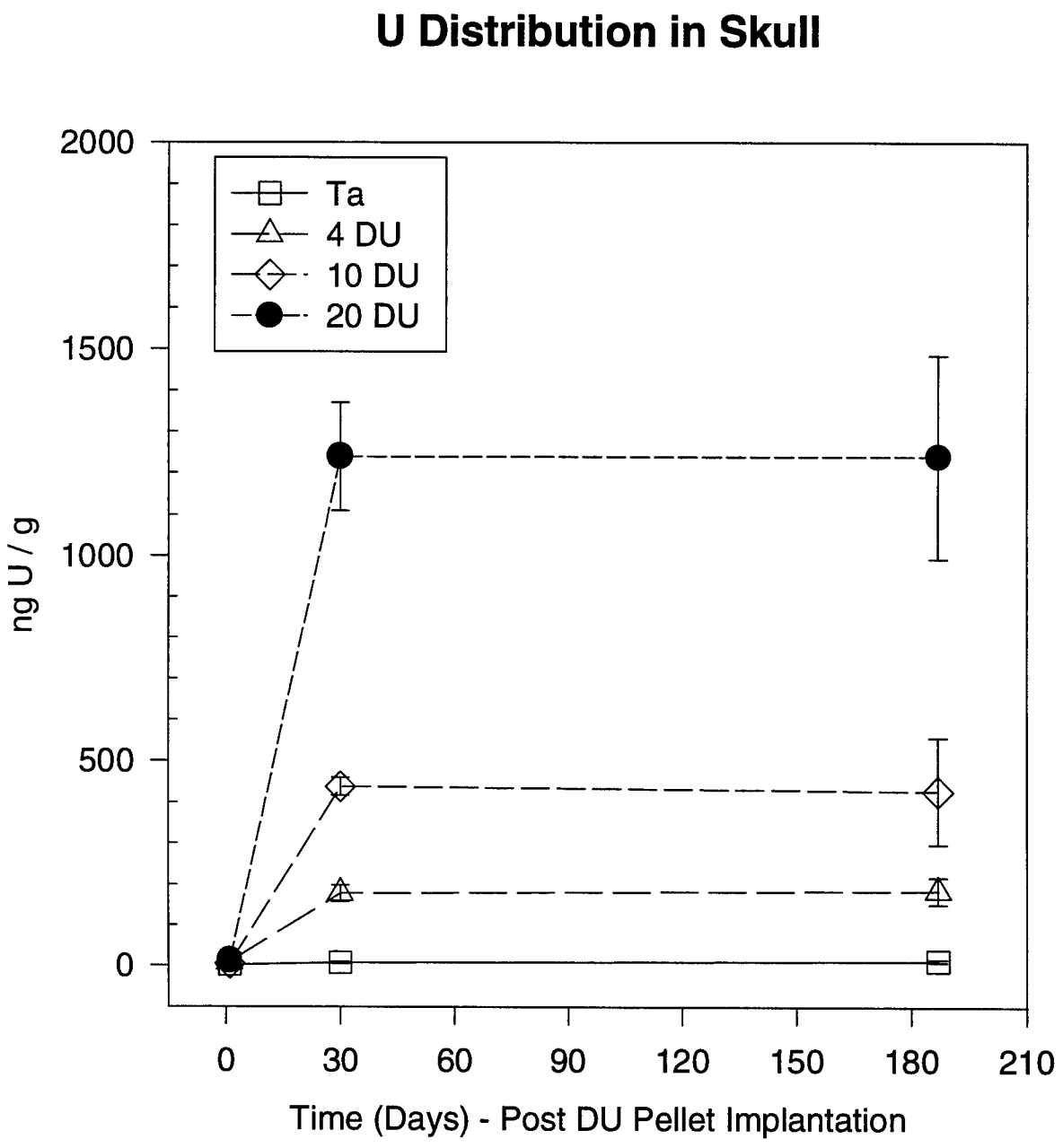

U Distribution in Skull
2000
1500
1000
500
0
ng U / g
Ta
4 DU
10 DU
20 DU
0
30
60
90
120
150
180
210
Time (Days) - Post DU Pellet Implantation

Figure 8

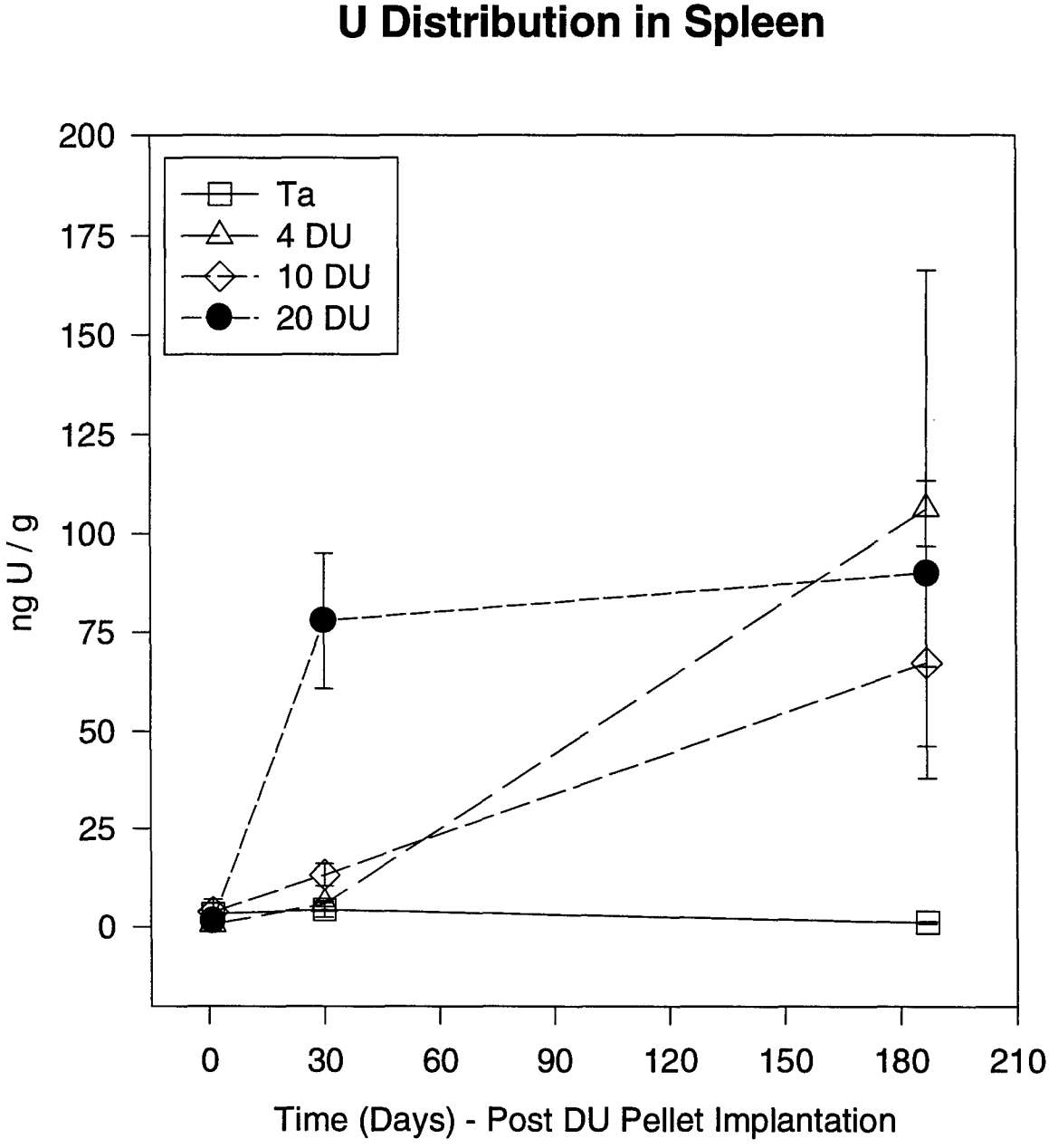

U Distribution in Spleen
200
175
150
125
100
75
50
25
0
ng U / g
Ta
4 DU
10 DU
20 DU
0
30
60
90
120
150
180
210
Time (Days) - Post DU Pellet Implantation

Figure 9

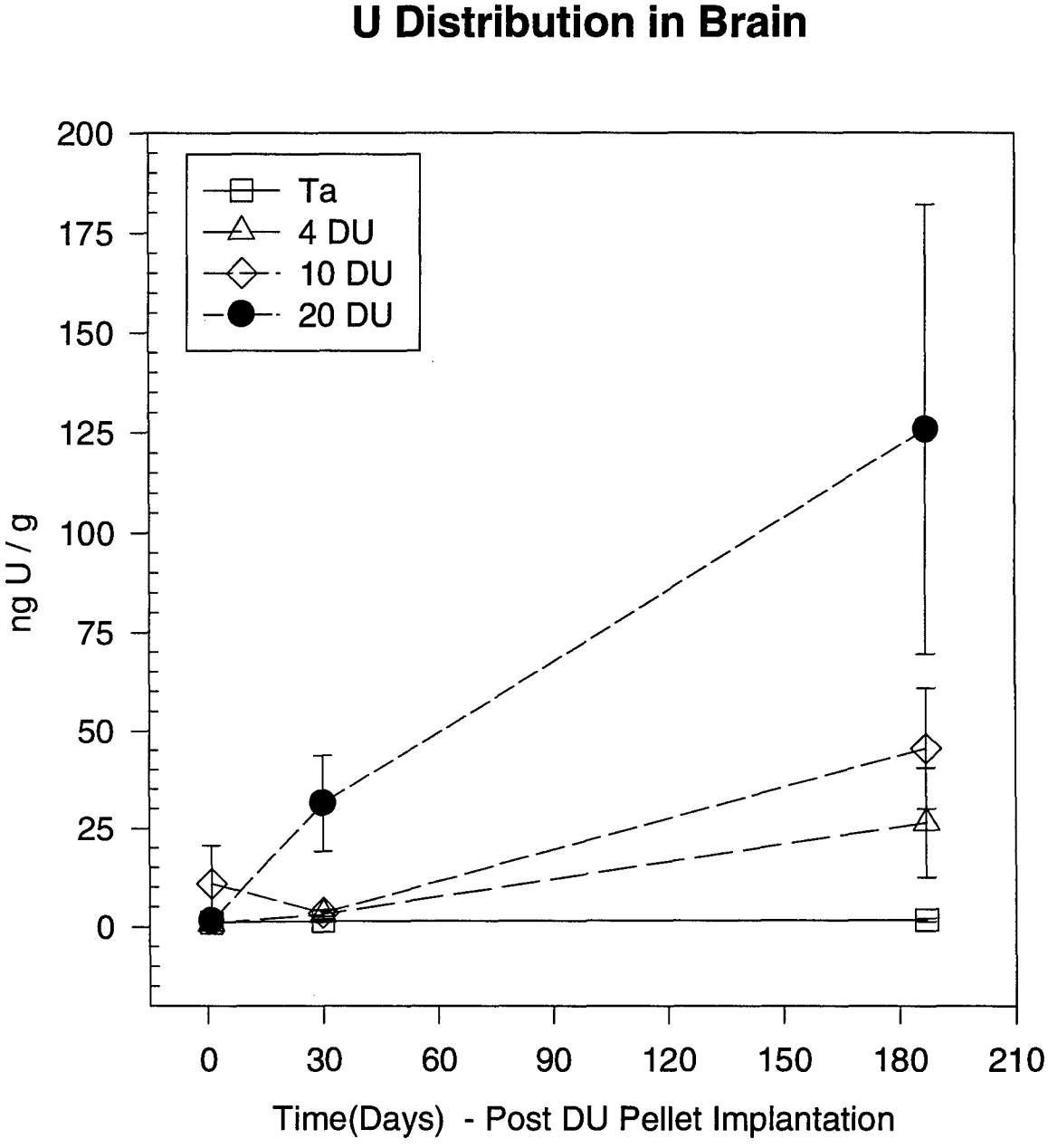

U Distribution in Brain
200
175
150
125
100
75
50
25
0
ng U / g
Ta
4 DU
10 DU
20 DU
0
30
60
90
120
150
180
210
Time(Days) - Post DU Pellet Implantation

Figure 10

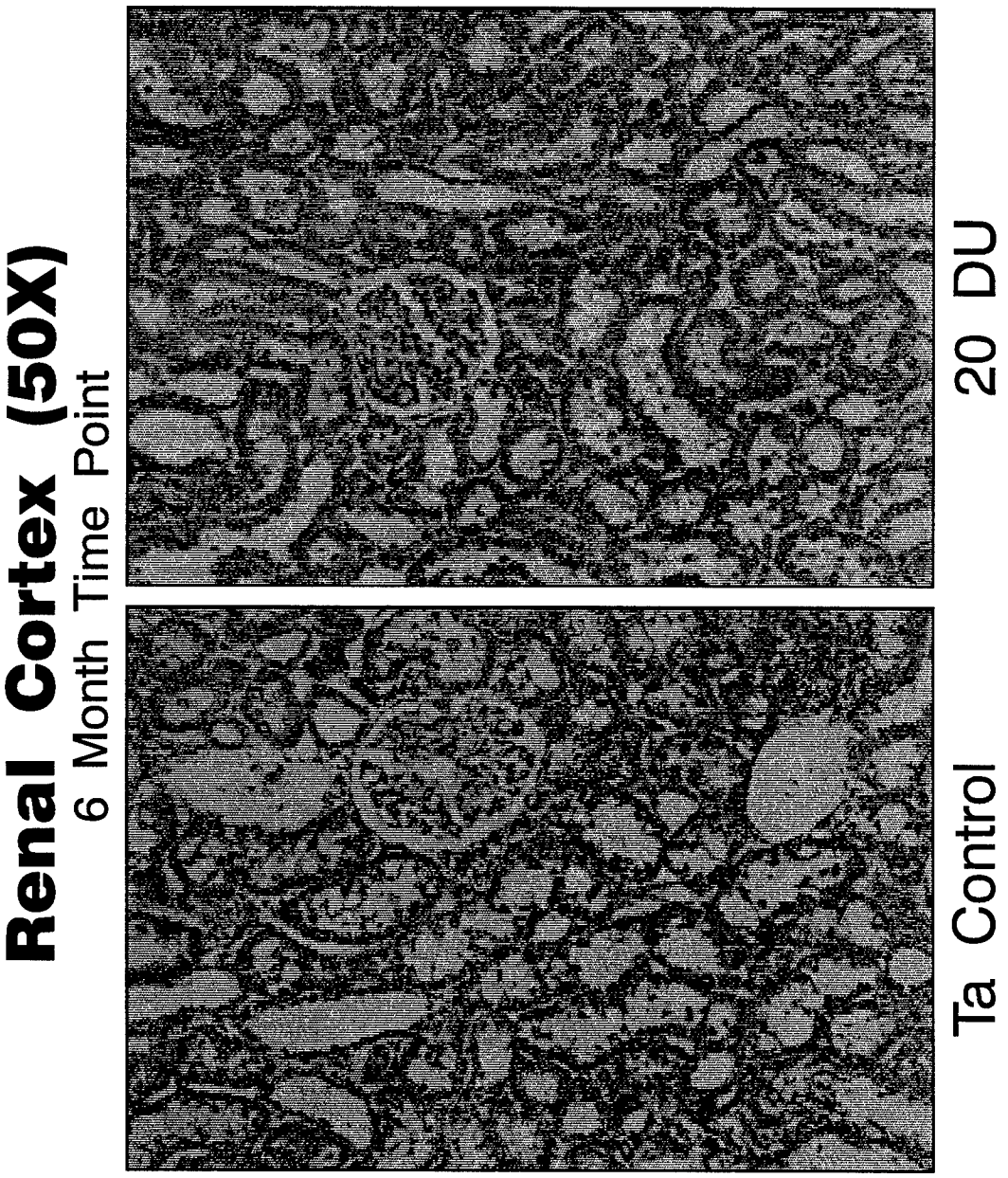


Figure 11

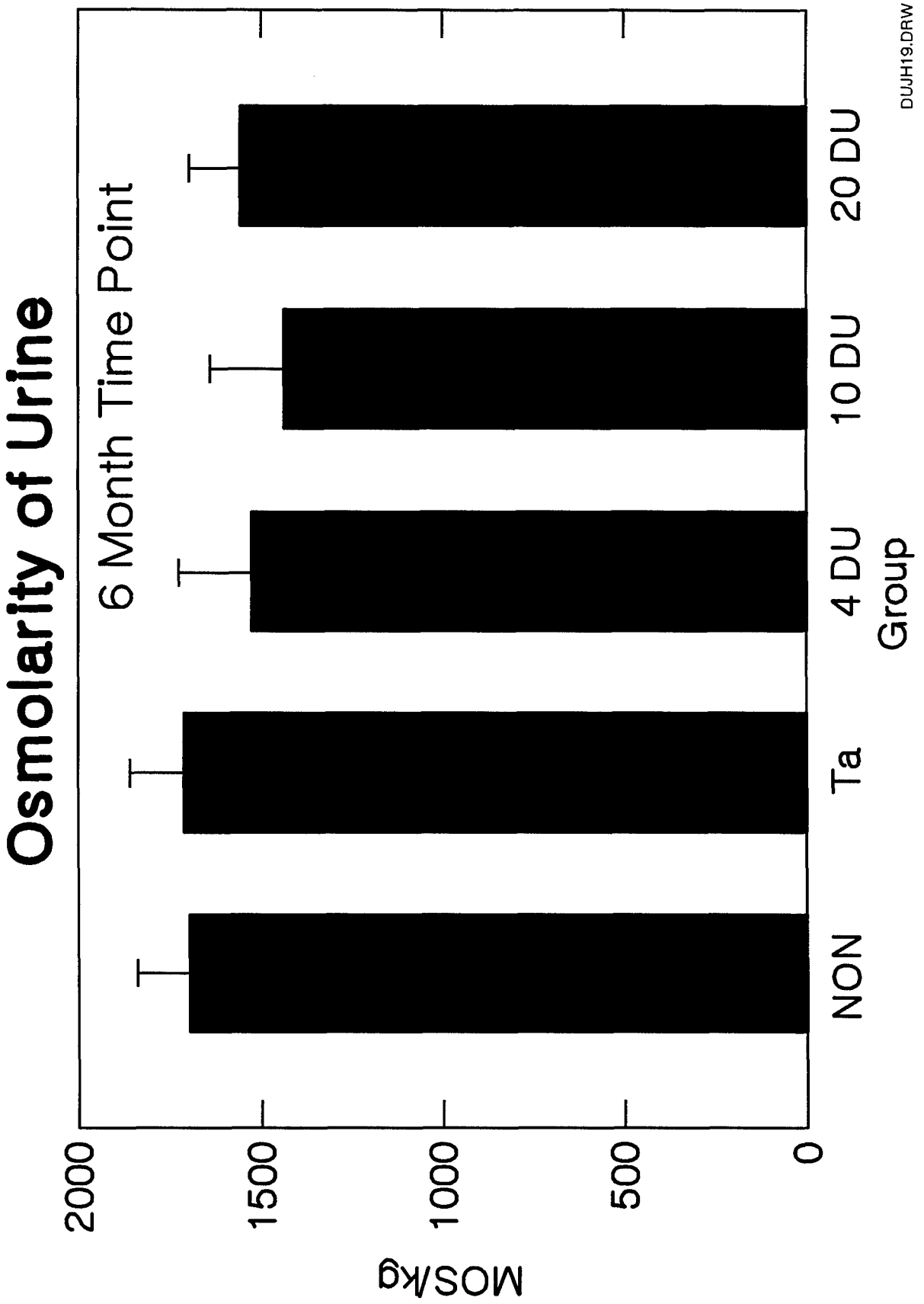

Osmolarity of Urine
6 Month Time Point
mOS/kg
2000
1500
1000
500
0
NON
Ta
4 DU
10 DU
20 DU
Group
DUJH19.DRW

Figure 12

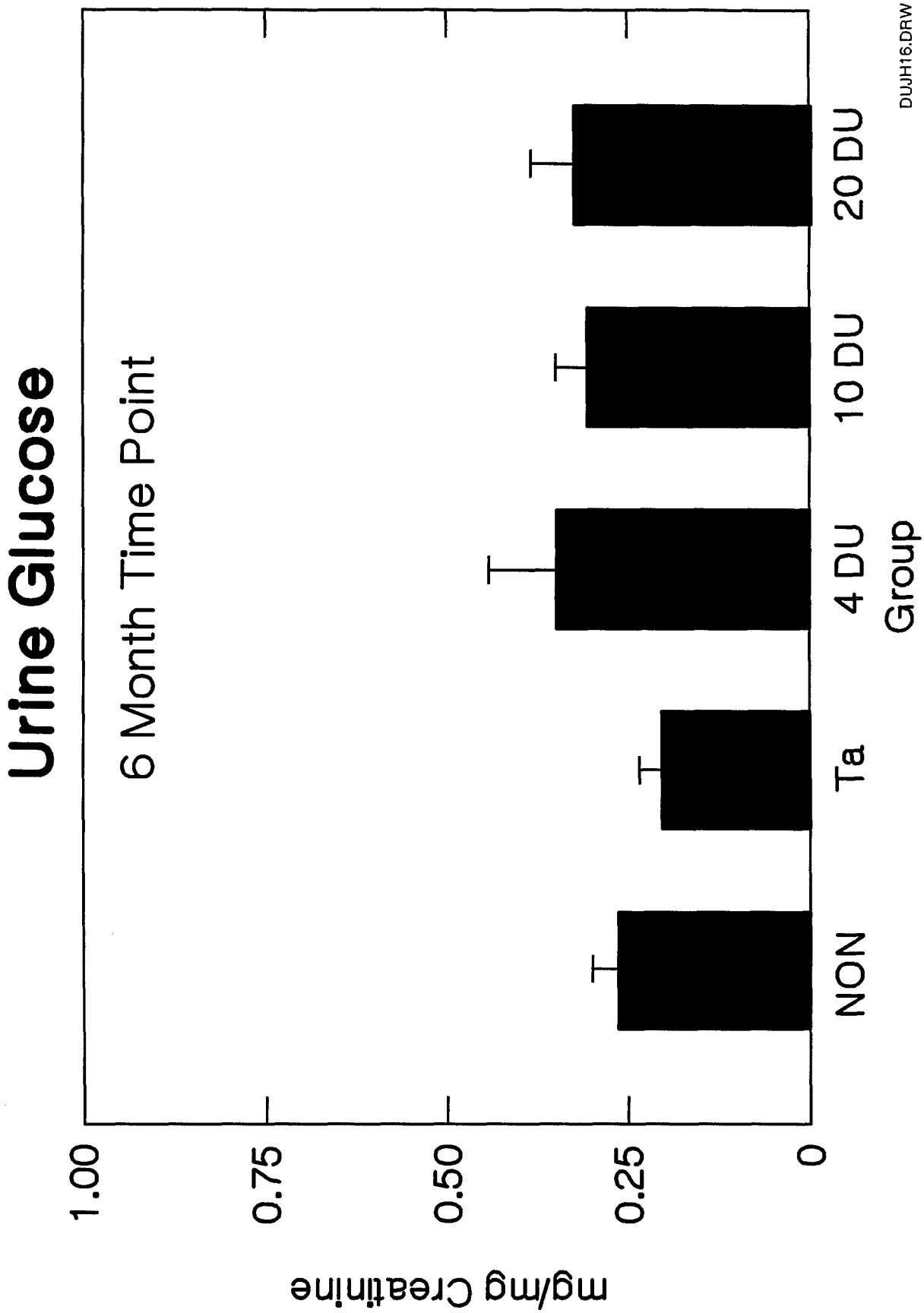

Urine Glucose
6 Month Time Point
mg/mg Creatinine
1.00
0.75
0.50
0.25
0
NON
Ta
4 DU
10 DU
20 DU
Group
DUJH16.DRW

Figure 13

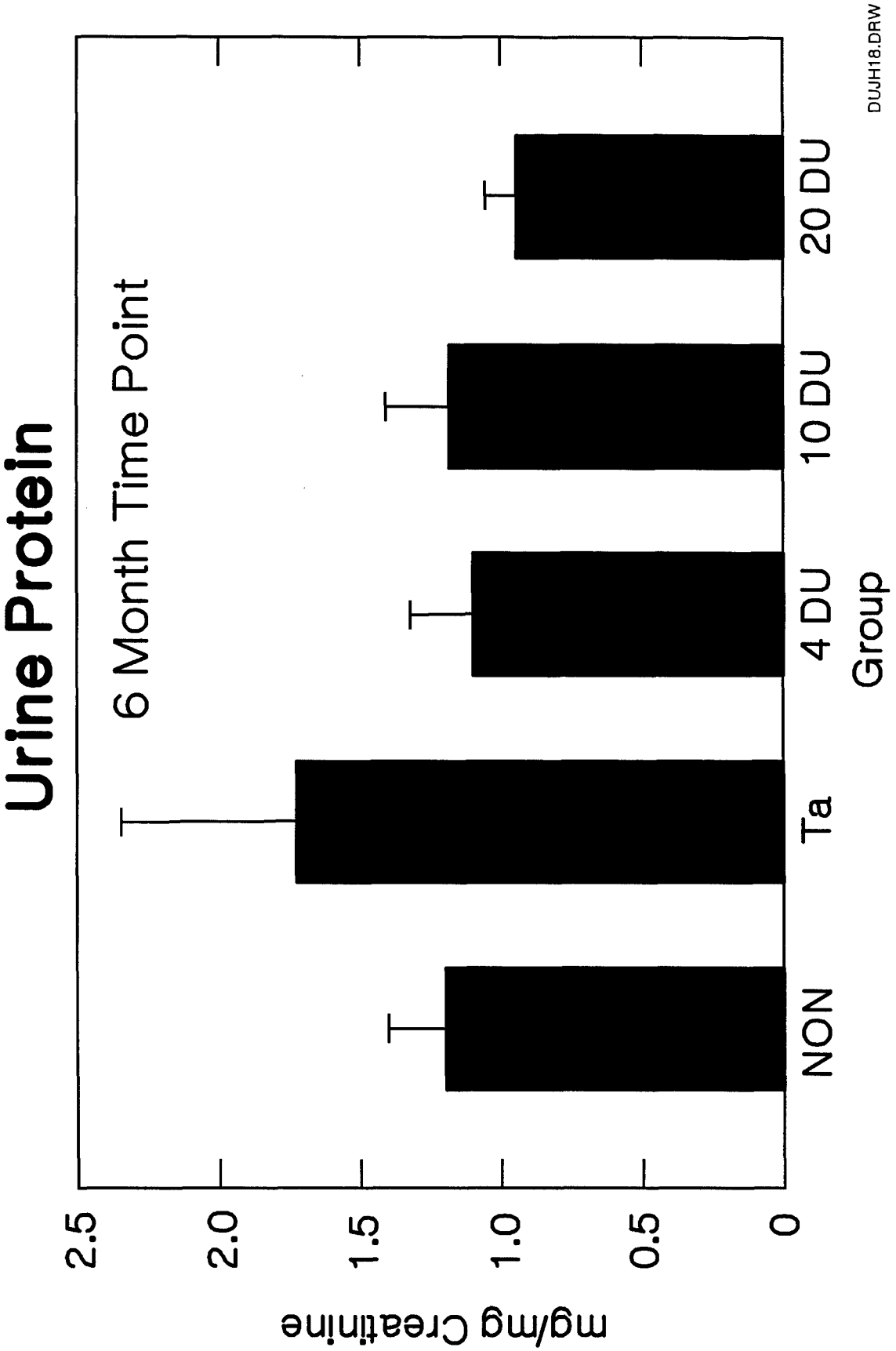

Urine Protein
6 Month Time Point
mg/mg Creatinine
2.5
2.0
1.5
1.0
0.5
0
NON
Ta
4 DU
10 DU
20 DU
Group
DUJH18.DRW

Figure 14

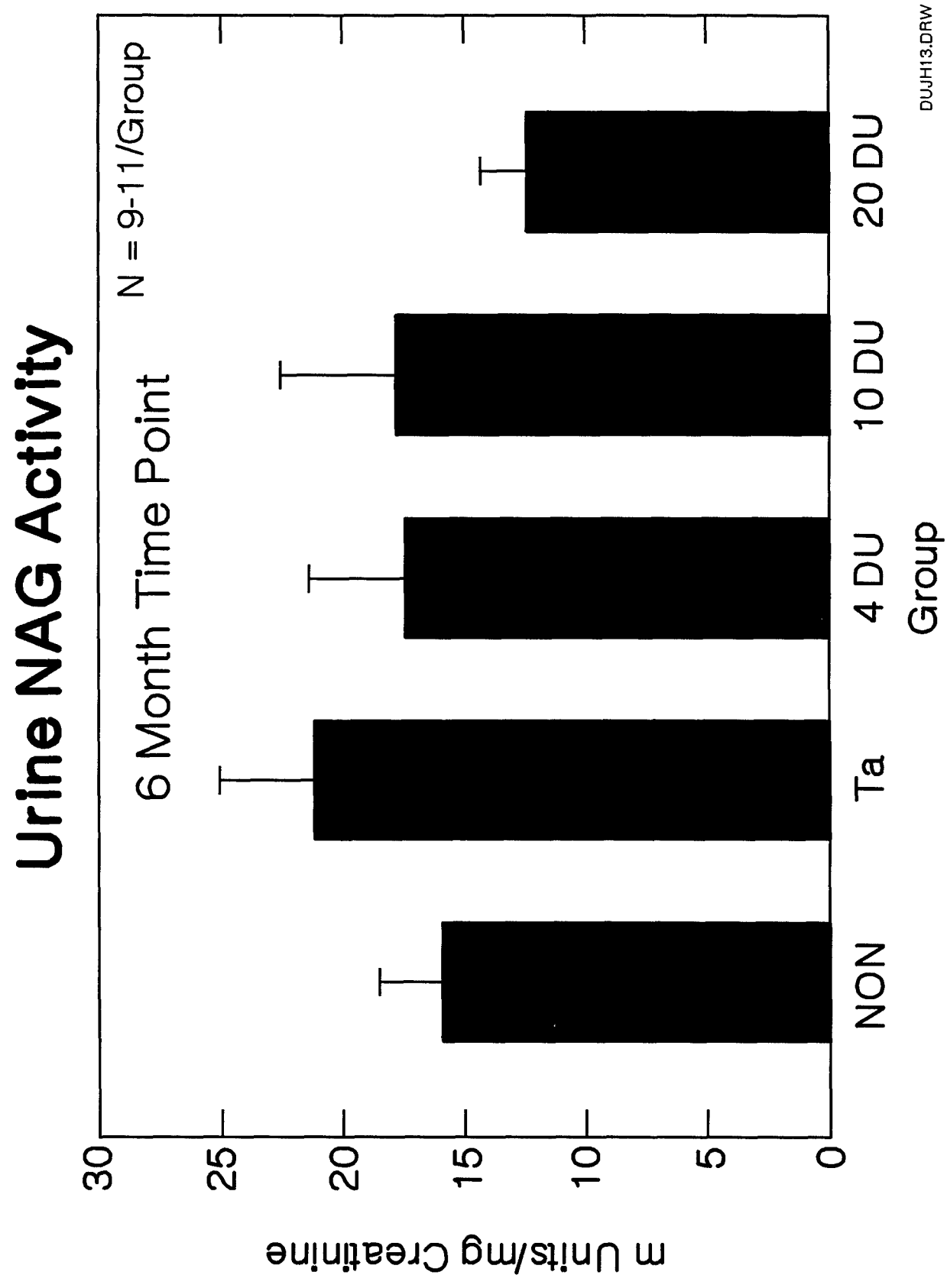

Urine NAG Activity
6 Month Time Point
N = 9-11/Group
m Units/mg Creatinine
30
25
20
15
10
5
0
NON
Ta
4 DU
10 DU
20 DU
Group
DUJH13.DRW

Figure 15

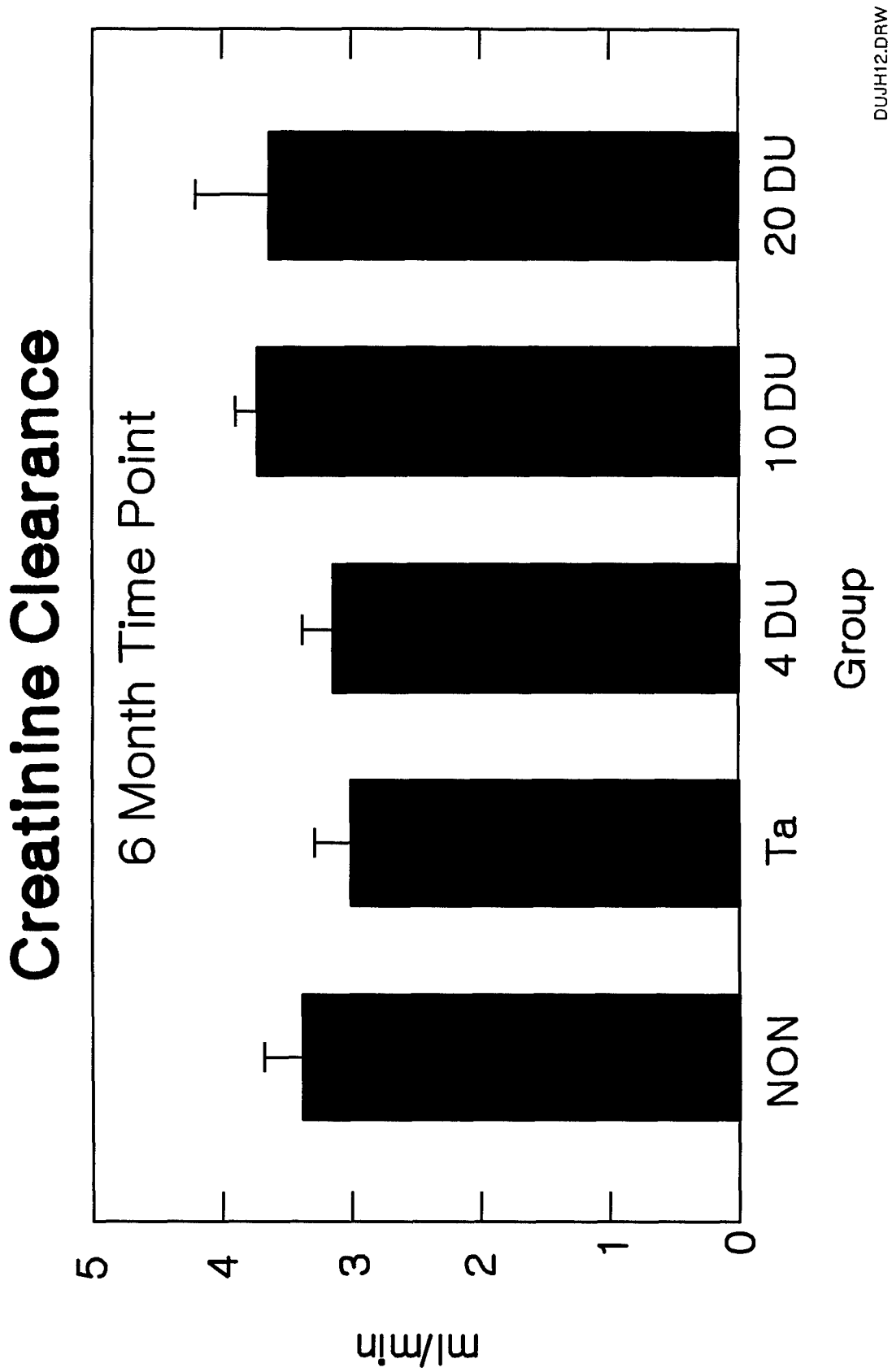

Creatinine Clearance
6 Month Time Point
ml/min
0
1
2
3
4
5
NON
Ta
4 DU
10 DU
20 DU
Group
DUJH12.DRW

Figure 16

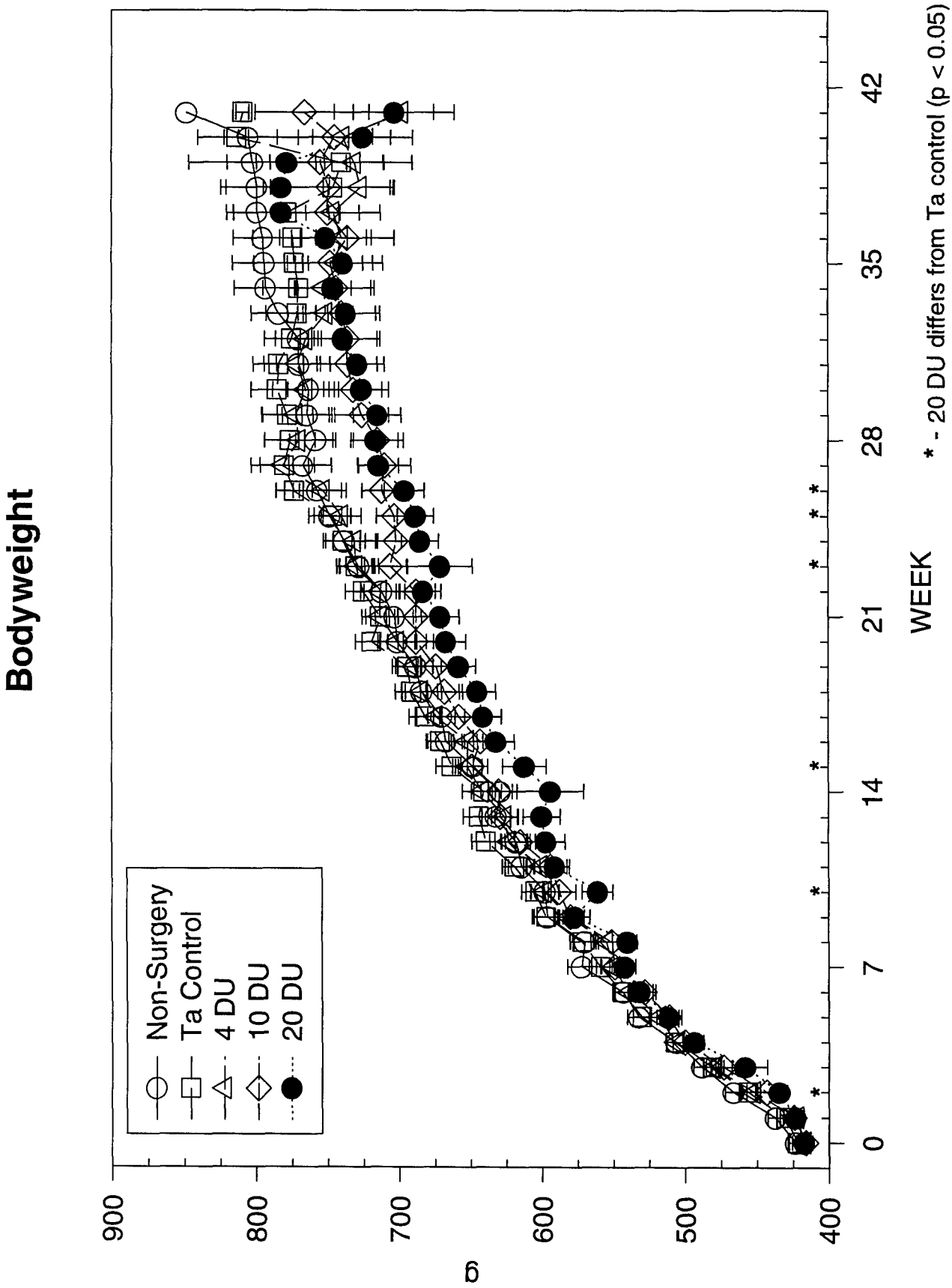

Bodyweight
g
900
800
700
600
500
400
0
7
14
21
28
35
42
WEEK
* - 20 DU differs from Ta control (p < 0.05)
Non-Surgery
Ta Control
4 DU
10 DU
20 DU

Figure 17

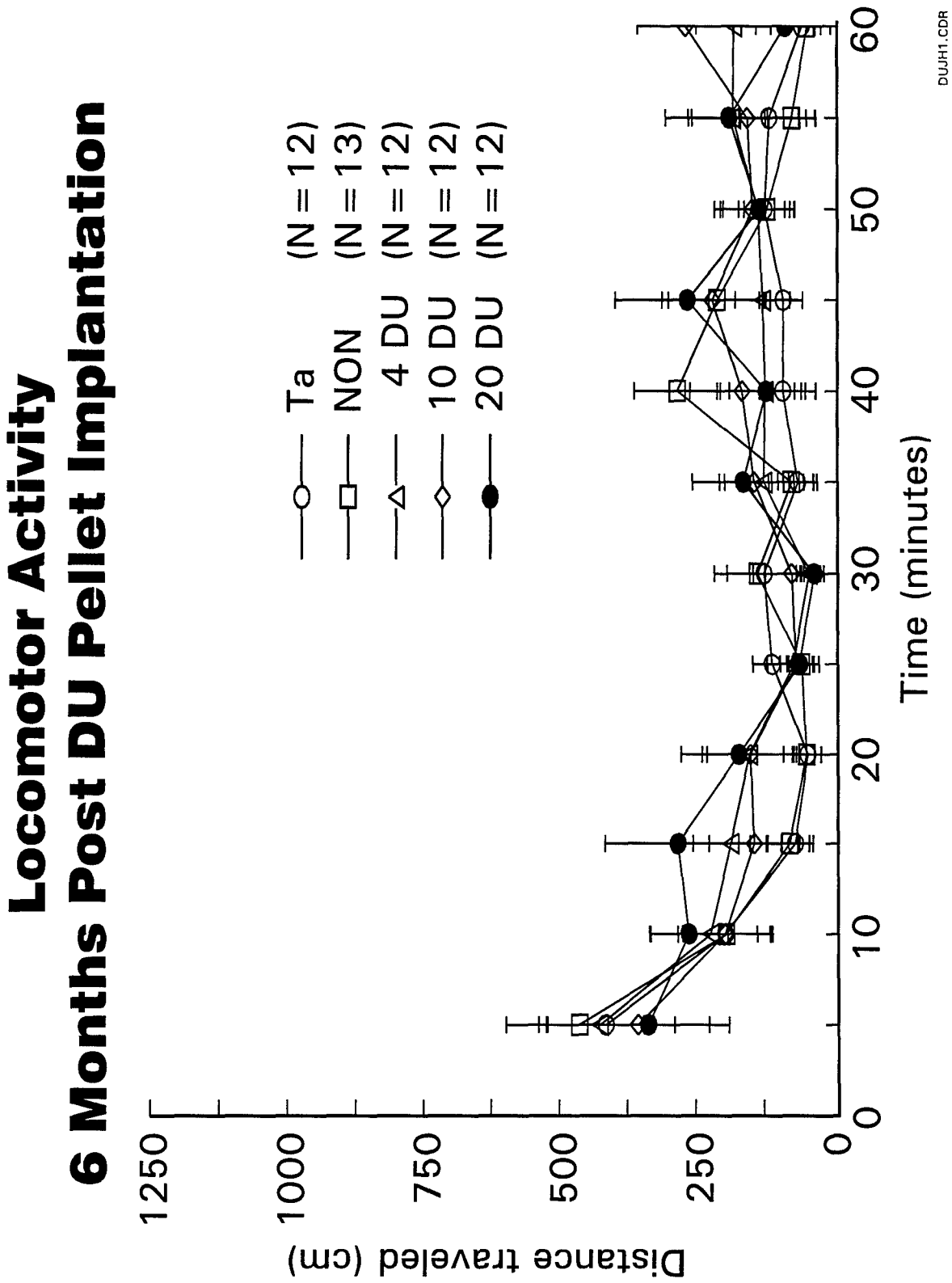
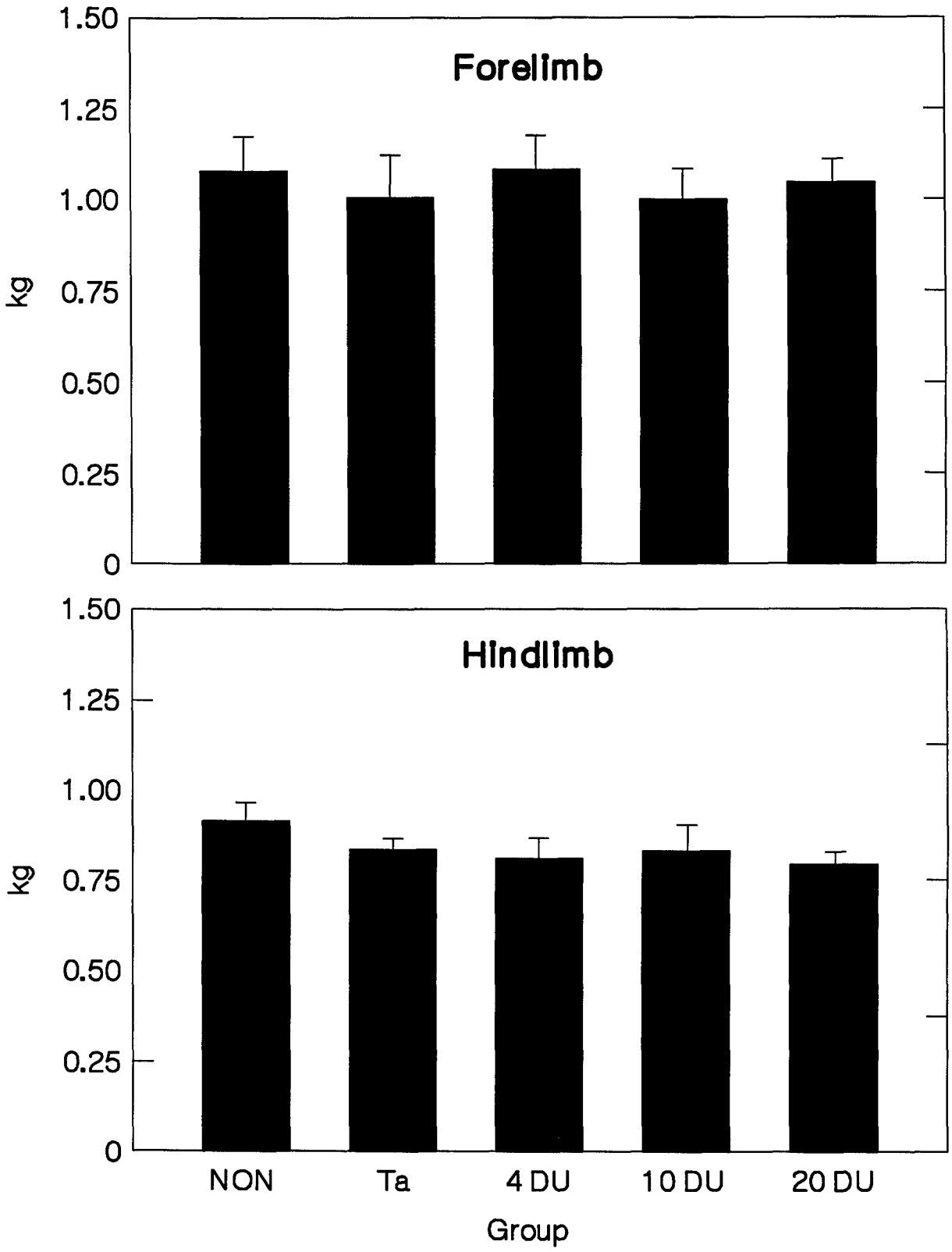

Locomotor Activity
6 Months Post DU Pellet Implantation
Ta (N = 12)
NON (N = 13)
4 DU (N = 12)
10 DU (N = 12)
20 DU (N = 12)
Distance traveled (cm)
0
250
500
750
1000
1250
0
10
20
30
40
50
60
Time (minutes)
DUJH1.CDR

Figure 18

Grip Strength

6 Month Time Point

N = 9-11/group



DUJH23.DRW

Figure 19

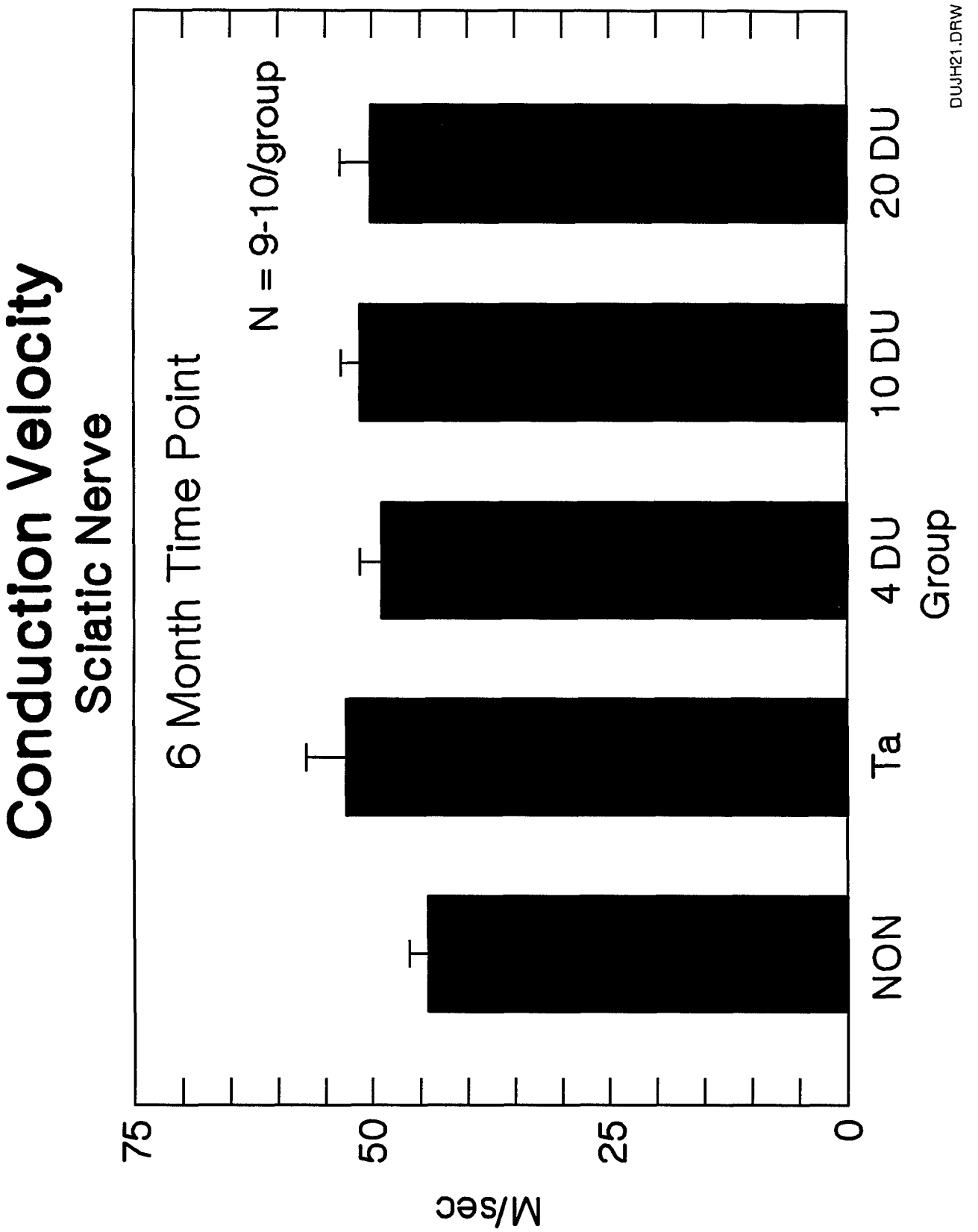

Conduction Velocity
Sciatic Nerve
6 Month Time Point
N = 9-10/group
M/sec
75
50
25
0
NON
Ta
4 DU
10 DU
20 DU
Group
DUJH21.DRW

Figure 20

Passive Avoidance

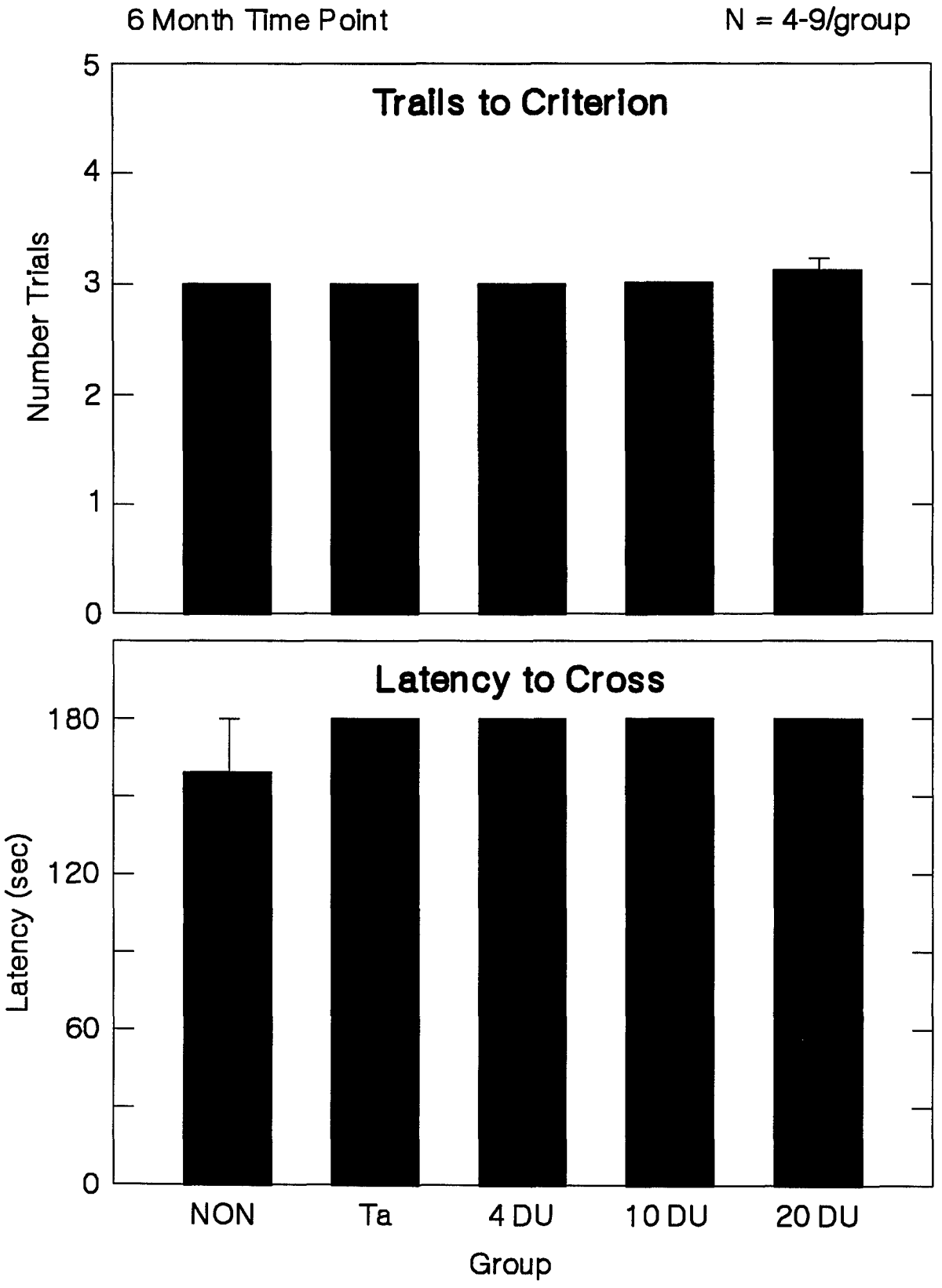
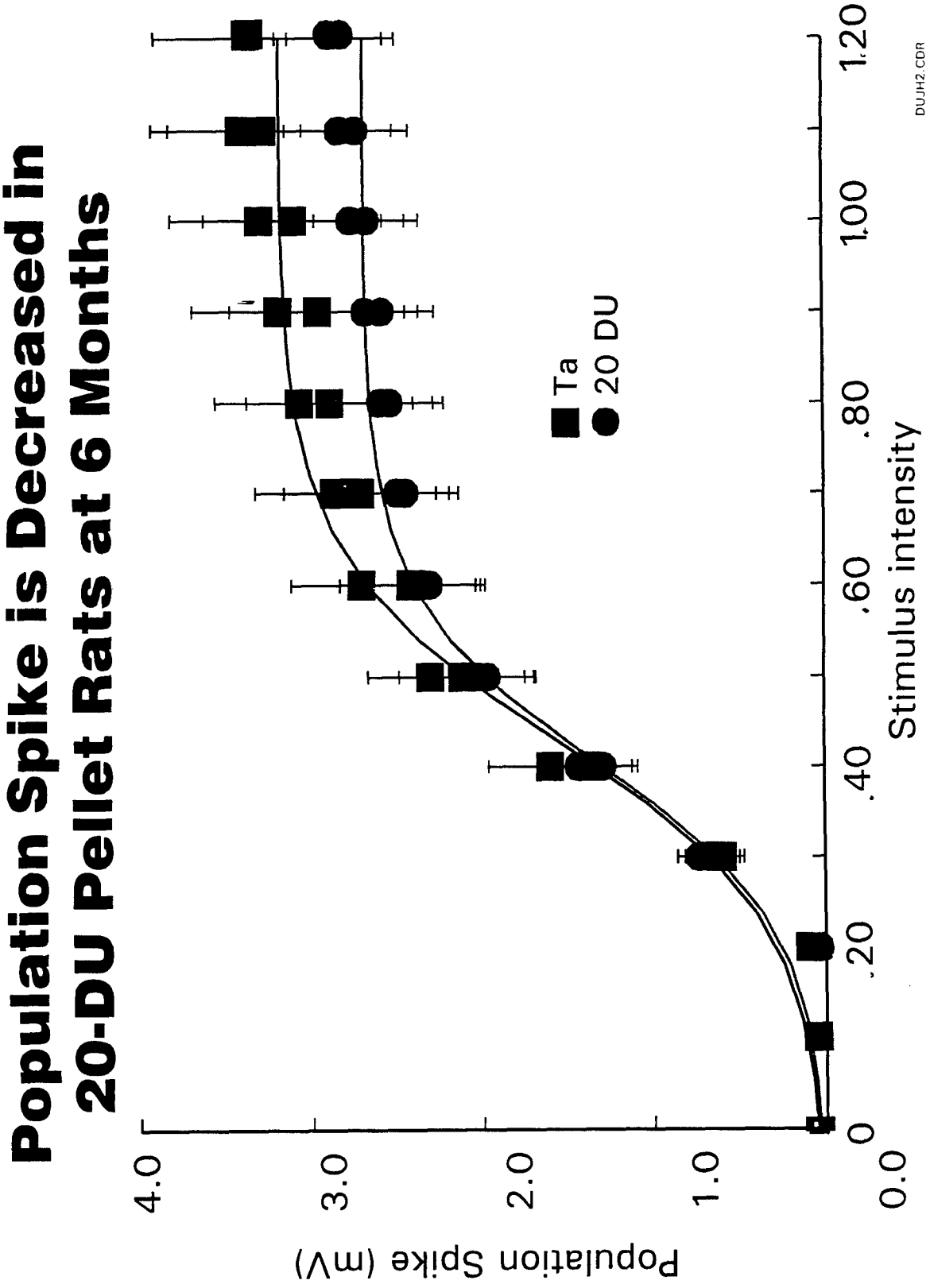


Figure 21


Population Spike is Decreased in 20-DU Pellet Rats at 6 Months
Ta
20 DU
Population Spike (mV)
4.0
3.0
2.0
1.0
0.0
0
.20
.40
.60
.80
1.00
1.20
Stimulus intensity
DUJH2.CDR

Received 2/8/00

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 20 Jan 00

MEMORANDUM FOR Administrator, Defense Technical Information Center, ATTN: DTIC-OCA, 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for Award No. 95MM5530. Request the limited distribution statement for Accession Document Number ADB227638 be changed to "Approved for public release; distribution unlimited." This report should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Virginia Miller at DSN 343-7327 or by email at virginia.miller@det.amedd.army.mil.

FOR THE COMMANDER:

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management